Development of a class II lanthipeptide production system

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Table of contents

Chapter 1

Introduction

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Abstract

Lantibiotics contain post-translational introduced (methyl)lanthionine residues, and are a group of peptides diverse in structure. Two distinct classes can be identified based on the structural organization of the enzymes introducing the characteristic thioether bonds, LanB and LanC in class I and the multifunctional enzyme LanM in class II. The antimicrobial activity of lantibiotics ranges from cell wall biosynthesis interference to membrane pore formation or a combination of these two activities. The main target is lipid II and lantibiotics appear in particular resilient to resistance mechanisms. In addition, the presence of the lanthionine bonds provide lanthipeptides with thermostability and proteolytic resistance. Lantibiotics form interesting candidates as novel antimicrobial substances. Here the mechanisms of lantibiotic biosynthesis, their mode of action and recent applications involving engineering are described.

Keynote abbreviations and terms

RiPP:	Ribosomally synthesized and post–translationally modified peptide
Lanthipeptide:	Lanthionine-containing RiPP
Lantibiotic:	Lanthionine-containing antibiotic peptide; lanthipeptide possessing antimicrobial activity (class I & II lanthipeptides)

1. Ribosomally synthesized and post-translationally modified peptides

Ribosomally synthesized and post–translationally modified peptides (RiPPs) (1) are natural products produced throughout the three kingdoms of life. It concerns a vastly growing group of peptides. In the recent proposed nomenclature by Arnison et al. (1), it is suggested to refer to the biosynthetic pathway of RiPPs as Post-Ribosomal Peptide Synthesis (PRPS).

RiPPs are genome encoded as precursor peptides. They consist of an N-terminal leader and a C-terminal core peptide (figure 1). A typical example of such a precursor peptide structure is found in the group of lanthipeptides. There are some exceptions where a leader-like peptide is found at the C-terminus like in the case of bottromycins. The precursor peptide can also contain a signal sequence at the N-terminus of the leader peptide. This is usually seen with RiPPs produced by eukaryotic cells. The signal sequence then directs the peptides to different cell-compartments, like the endoplasmic reticulum, where modification and secretion takes place. The precursor peptide can also encode an additional recognition sequence at the C-terminus, such as motifs that direct peptide cyclization as found in cyanobactins and amatoxins (1). The N-terminal leader peptide serves as a recognition region for the post-translational modification enzymes, and for the export and immunity proteins (1, 2). Although the exact mechanism of leader recognition is not known, most leader peptides form an α-helix that is believed to bind to the associate biosynthetic proteins (3-5).

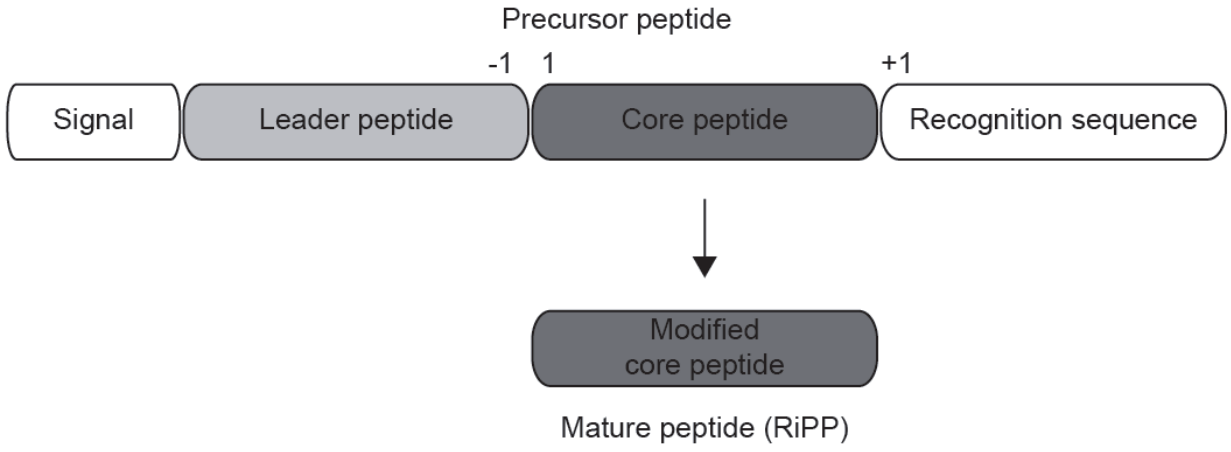

Figure 1: Schematic representation of the RiPP precursor peptide, which is post-translationally modified and after proteolysis and secretion yields the mature peptide. (Made after the proposed nomenclature described by Arnison et al. (1))

2. Lanthipeptides

An example of peptides belonging to the category of RiPPs are lanthipeptides, lanthionine-containing peptides. Known lanthipeptides display a diverse range of activities. They can be morphogenetic (6), antiviral (7), antiallodynic (8), or antimicrobial. When lanthipeptides possess antimicrobial activity they are called lantibiotics, lanthionine-containing antibiotic peptides (9). Lanthipeptides contain unusual *meso*-lanthionine (Lan) residues, but in addition may also contain 3-methyllanthionine (MeLan) residues. These (methyl)lanthionine bonds are believed to give lanthipeptides thermostability and proteolytic resistance, but they are also critical for their activity. A lanthionine is composed of two alanine residues that are cross-linked on their β-carbon via a thioether bond. The methyllanthionine structure contains an extra methyl group. The Lan and MeLan residues are introduced into the precursor peptide in two enzymatic steps. Serine and threonine, respectively, are dehydrated to form dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Hereafter the dehydrated residues are coupled to a cysteine via Michael-type addition, to form a thioether crosslink. Based on the modification enzymes that introduce the (methyl)lanthionine residues the lanthipeptides can be divided into four different classes (10) (figure 2). The well characterized class I and II lanthipeptides, that mainly comprise lantibiotics and therefore also known as class I and II lantibiotics. And the more recently discovered class III and IV lanthipeptides.

2.1 Class I lanthipeptides

In class I lanthipeptides/lantibiotics, the (methyl)lanthionine residues are formed by two distinct enzymes, a dehydratase (LanB) and a cyclase (LanC). Another characteristic of class I lanthipeptides is their elongated and flexible structure (figure 3). This secondary structure plays an important role in the antimicrobial effect of class I lantibiotics by binding to lipid II and in the pore formation (11, 12). Peptides belonging to class I lanthipeptides include subtilin, epidermin, Pep5 and the well-known lantibiotic nisin. Nisin is produced by *Lactococcus lactis* and was discovered in 1927 (13). It is one of the few commercial used lanthipeptides and shows activity against bacteria involved in food spoilage, like *Bacillus cereus* and *Listeria monocytogenes*. Since nisin has a food-grade status it is applied as a preservative (E234, Nisaplin®) in the food industry for example in dairy, bakery, vegetables, meat and fish products (14).

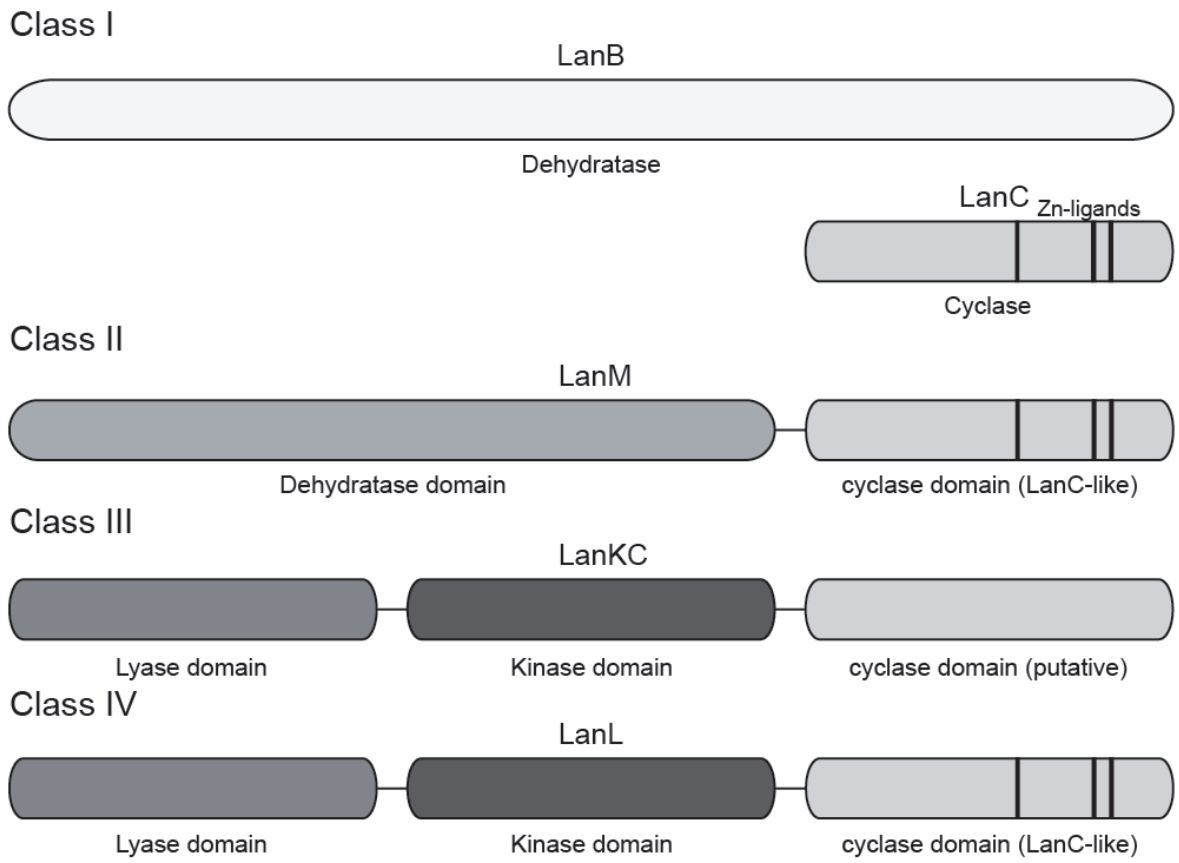

Figure 2: Schematic representation of the four lanthipeptide classes based on the lanthionine introducing modification enzymes. The dark lines in LanC and LanC-like cyclase domains represent the conserved Zn-ligands. (made after the examples from Yu et al. and Zhang et al. (51, 129))

2.2 Class II lanthipeptides

In class II lanthipeptides/lantibiotics there is a single multifunctional lanthionine synthetase (LanM) that carries out both the dehydration and cyclization reactions. LanM contains two domains, an N-terminal dehydration domain and a C-terminal cyclase domain. The N-terminal dehydration domain does not show any homology to LanB of the class I lantibiotics. However, the C-terminal cyclase domain shows about 25% sequence identity with LanC (10), including the conserved zinc-binding residues (15).

Unlike class I lantibiotics, class II peptides generally have a more globular structure. Another characteristic feature of class II lantibiotics is the presence of a bifunctional transporter encoded within the gene cluster. This transporter

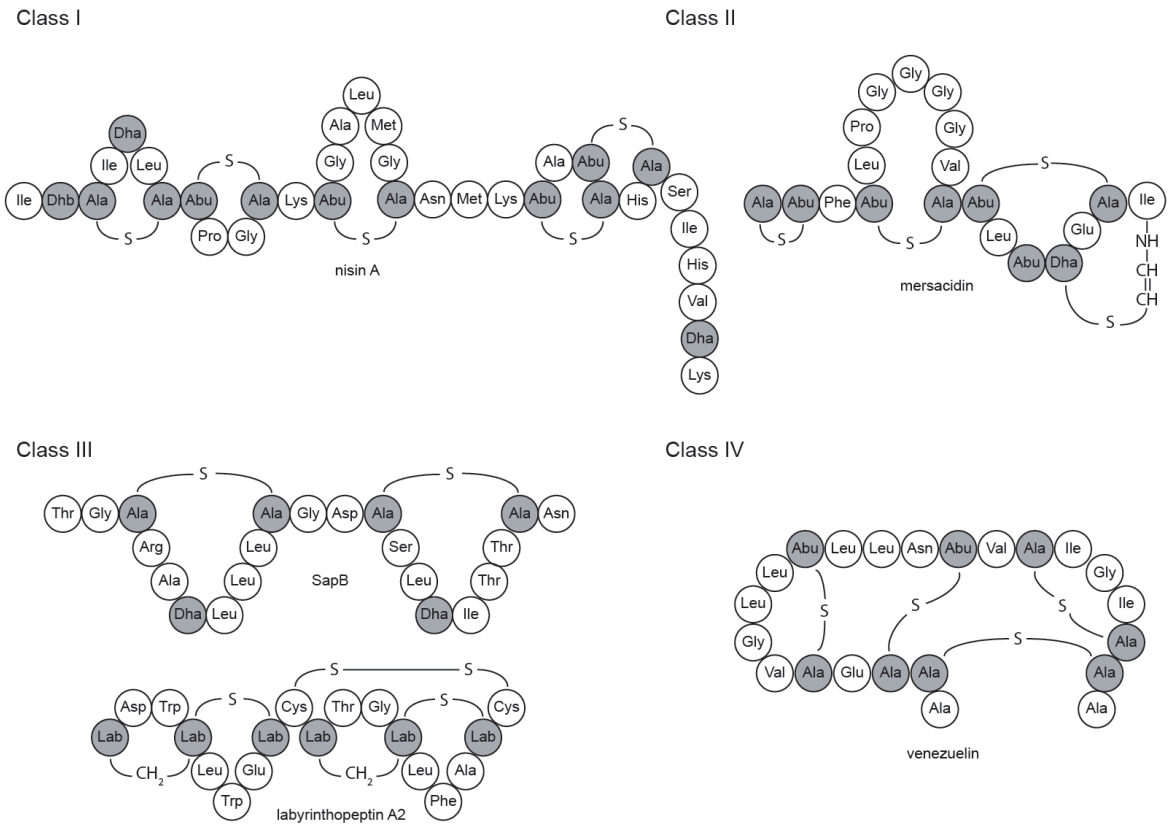

Figure 3: Representative examples of lanthipeptides belonging to four different classes. This includes the elongate structure of class I lanthipeptides and the labionin structures characteristic for class III lanthipeptides. The structure of venezuelin, class IV, is putative (21, 45).

fulfills a dual role, i.e., transporting the peptide across the membrane and removal of the leader peptide from the lanthipeptide through the activity of an N-terminal peptidase domain. Within the class II lantibiotics there is an additional group termed the two-component lantibiotics. These lantibiotics consist of two structural peptides that are both necessary for antimicrobial activity. Both are modified by individual LanM enzymes, but transported by a single LanT which in addition cleaves the leader peptides. An example of such a two–component lantibiotic is the well-described lacticin 3147 (16, 17).

2.3 Class III lanthipeptides

Class III lanthipeptides, e.g. SapB and Labyrinthopeptins, lack antimicrobial activity. Instead these peptides have morphogenetic and signaling functions. Like class II peptides, class III peptides are modified by a single multifunctional

modification enzyme termed LanKC. This enzyme contains three domains: an N-terminal lyase domain, a central kinase domain and a C-terminal cyclase domain. The lyase domain and the kinase domain, carry out the dehydration of serine and threonine residues, while the cyclase domain forms the distinct (methyl)lanthionine rings. The cyclase domain of LanKC does not contain the characteristic zinc-binding motifs found in LanC and LanM. However, it does show homology to other cyclase domains (18). In addition to the lanthionine bond, a group of the class III enzymes also form the so-called labionin structures (figure 3), known from labyrinthopeptins (8). The labionin (Lab) structure is formed by the addition of a second Dha to an enolate intermediate, formed by the addition of a Cys thiol to Dha (19, 20) (figure 6). Besides morphogenic and signaling functions, class III peptides may be used as therapeutic agents. The labyrinthopeptin A2 peptide is currently under development as an antiallodynic drug (8).

2.4 Class IV lanthipeptides

A fourth class, class IV lanthipeptides, has been assigned recently through the discovery of the lanthipeptide venezuelin produced by *Streptomyces venezuelae* (21). While the multifunctional lanthionine synthetase, LanL, resembles the class III enzyme, it differs at the C-terminal cyclase domain. The enzyme contains three distinct domains, an N-terminal lyase domain, a central kinase domain and a C-terminal cyclase domain. While the lyase domain and the kinase domain show homology towards the corresponding domains of the enzyme of class III peptides, the cyclase domain of LanL is homologous to LanC (21). It contains the characteristic zinc-binding motifs absent in the class III lanthionine synthetase.

3. Lantibiotic gene clusters

Lanthipeptides are encoded in gene clusters. These gene clusters can be found on plasmids, conjugative transposable elements or on the chromosome of the producing organism (22). The generic locus symbol that has been assigned to the genes in the lanthipeptides gene clusters is *lan*, but to distinguish between individual gene clusters of lanthipeptides more specific names are used such as for nisin or mersacidin, where *nis* or *mrs* is used, respectively. Examples of lantibiotic gene clusters are shown in figure 4. These gene clusters contain genes

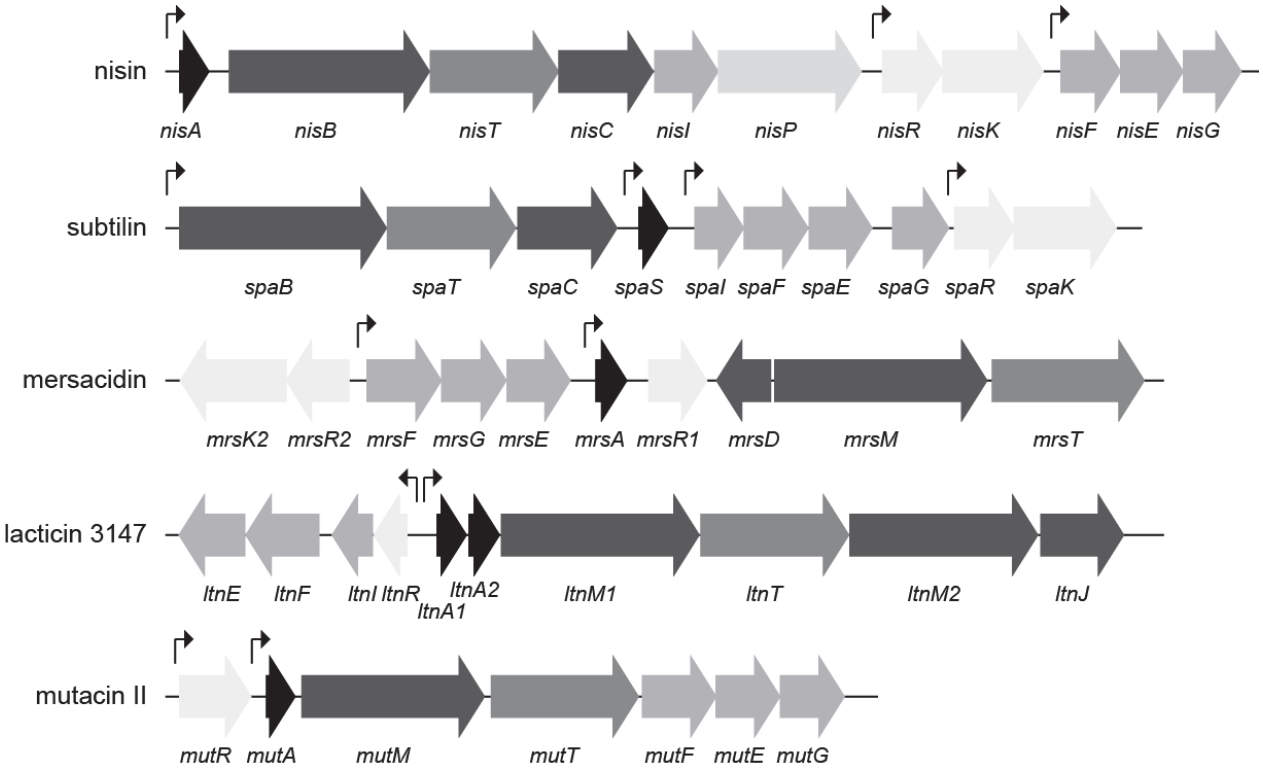

Figure 4: Lantibiotic gene clusters involved in the biosynthesis of nisin, subtilin, mersacidin, lacticin 3147 and mutacin II. Promoters, where known, are indicated by small arrows.

for lantibiotic biosynthesis, regulation, self-immunity, transport and processing.

The gene encoding the precursor peptide is labeled *lanA*, for instance *nisA*, *mutA* or for the two-component lantibiotic lacticin 3147, *ltnA1* and *ltnA2*. The precursor encoding genes are usually clustered in one operon with the modification enzymes designed genes: *lanB* for the lantibiotic dehydratase, *lanC* for the cyclase or *lanM* for the bifunctional lanthionine synthase of class II lantibiotics. In addition, lantibiotics can undergo further post translational modifications as for instance decarboxylation of the C-terminus by LanD, or the conversion of L-serine to D-alanine by LanJ. To transport the peptide across the cytoplasmic membrane a dedicated transporter is encoded in the gene clusters, termed *LanT*. In class II lantibiotics this transporter contains a peptidase domain, which will cleave off the leader peptide during or prior to transport. Since class I lantibiotic transporters do not contain this peptidase domain their gene cluster generally encodes an extracellular serine protease, LanP. However, not all lantibiotic gene clusters encode a dedicated protease, for instance the gene is missing in the gene cluster of subtilin. Studies on the maturation of subtilin

revealed it was matured by nonspecific extracellular serine proteases produced by subtilin producing and non-producing strains (70, 71). Proteins involved in lantibiotic biosynthesis, transport and processing will be described in more detail in section 4.

3.1 Lantibiotic regulatory genes

Regulatory genes, *lanR* and *lanK*, are also encoded in the lantibiotic gene clusters. *LanK* generally encodes a receptor-histidine kinase and *lanR* a transcriptional response regulator. The best described lantibiotic regulation systems are that of nisin and subtilin (23, 24). Their biosynthesis is autoregulated by a two-component regulatory mechanism, also described as quorum-sensing systems. For example in the biosynthesis of nisin, the presence of low concentrations active lantibiotic will be detected by the receptor-histidine kinase present at the cell surface. Binding of lantibiotic to NisK will trigger the autophosphorylation of a histidine residue, a signal cascade is initiated were the phosphoryl group is transferred to a aspartate residue on NisR. Next, NisR will bind the *nisA* and *nisF* operators, and transcription of *nisABTCIP* and *nisFEG* operons are initiated. The *nisR* promotor regulates the *nisRK* genes, however, its regulation is not known (25). The regulation seems to be related to the growth stage of the producing strain. Nisin production starts during the mid-exponential growth phase and peaks at the transition from log- to stationary-phase (23). Regulation of subtilin biosynthesis involves multiple environmental signals before production is initiated. As described for nisin, subtilin biosynthesis is dependent on the presence of SpaK and SpaR. The transcription of the *spaRK* genes is dependent on the alternative sigma factor, σ^H, which in turn is inhibited by the transition state regulator AbrB (24). From the autoregulatory systems of nisin and subtilin heterologous gene expression tools have been constructed, namely the well-known nisin-controlled gene expression (NICE) and subtilin-regulated gene expression (SURE) systems (26, 27). A typical two-component regulatory system is also present in the mersacidin gene cluster, *mrsR2* and *mrsK2*. However, these genes only regulate transcription of the immunity genes, *mrsFGE*, when mersacidin is present extracellular. In addition, the gene cluster also encodes a second transcriptional response regulator, *mrsR1*, which is necessary for transcription of the mersacidin biosynthesis genes. A corresponding receptor-histidine kinase has not been found. MrsR1 does not seem to be

autoregulated and it is unknown what triggers the transcription of the mersacidin biosynthesis genes (28). These so-called orphan regulators are not uncommon in lantibiotic gene clusters. For example, EpiQ of the epidermin gene cluster (29), LtnR of the lacticin 3147 gene cluster (30), and MutR of the mutacin II gene cluster (31). Biosynthesis is also closely regulated by specific cellular events and culture conditions. For example, the sigma factor σ^H controlling SpaKR in subtilin production is only formed during stationary phase (24), or a drop in pH induces RcfB which in turn activates lacticin 481 production (32).

3.2 Lantibiotic immunity genes

Since lantibiotics are produced and act against closely related strains, self-protection is needed. Therefore, the lantibiotic gene clusters encode self-protection/immunity proteins. There are two mechanisms that can be employed for self-immunity. *LanI* encodes an immunity protein and *lanFE(G)* encode a ABC transporter. Both LanI and LanFE(G) can function on their own as immunity mechanisms or the proteins can co-operate to mediate immunity. The immunity ABC transporters consists of two or three subunits. LanF is the ATPase domain and LanE and LanG are integral membrane domains. Lantibiotics that have reached the cytoplasmic membrane, before or during pore formation, are excreted into the environment by such ABC transporters. Especially, the gene clusters of lipid II targeting lantibiotics contain genes encoding the immunity ABC transporter (33).

LanI proteins show little similarity towards each other, suggesting diverse immunity mechanisms. LanI can bind the produced lantibiotic prohibiting it from binding to the target or it may shield the membrane target from the lantibiotic. NisI is a hydrophilic lipoprotein with an N-terminal lipid moiety that anchors the protein to the cell membrane and interacts with nisin. However, not all NisI contains this lipid membrane anchor and a fraction of the protein is secreted from the cell. Extracellular NisI will aggregate nisin (34) and thereby inactivate the lantibiotic. Although NisI and NisFEG provide two immunity mechanisms, complete immunity is only provided when all nisin immunity genes are expressed. The subtilin gene cluster also contains both immunity mechanisms. SpaFEG functions like NisFEG in lantibiotic excretion. However, SpaI seems to employ a different method compared to NisI. SpaI is mostly a hydrophilic protein

with an N-terminal lipid anchor. SpaI sequesters subtilin in the cytoplasmic membrane preventing co-localization and pore formation by the peptides (35).

The gene cluster of Pep5 contains the immunity protein, PepI, but lacks an immunity ABC transporter. PepI is relatively small with a hydrophobic N-terminus and a hydrophilic C-terminus. PepI is located at the membrane-cell wall interface and thought to shield the targets, possibly teichoic or lipoteichoic acids, from Pep5 thereby preventing pore formation (36). Another example of a LanI peptide is that of lacticin 3147. LtnI contains three predicted hydrophobic regions of about 20 amino acids suggesting a transmembrane localization. In addition, it contains a leucine zipper motif at the C-terminus through which dimerization is possible (37). LtnI binds and aggregates lacticin 3147, in that way, it prevents membrane insertion and pore formation. In addition, it may also shield lipid II as target of lacticin 3147.

A third gene, *lanH*, has been identified to play a role in the immunity systems. LanH is an accessory factor which is involved in the assembly of the immunity ABC transporter, e.g. EpiH of epidermin. A second example of a LanH is that of NukH, of the nukacin ISK-1 gene cluster. The structure of NukH resembles that of LtnI, with three membrane spanning domains (38), and it was proposed to bind nukacin ISK-1 and thereby inactivate the lantibiotic (39). Moreover, the nukacin ISK-1 gene cluster also contains the immunity ABC transporter. Expression studies proposed a cooperative action between NukH and NukFEG, which would be more in line with the accessory function in assembly (38).

Summarizing, different immunity mechanisms have been proposed for LanI, LanFE(G) and LanH. The LanFE(G) proteins seem to be more conserved in structure and proposed mechanism of immunity throughout lantibiotics. For LanI, different mechanisms have been proposed that are specific for the lantibiotic produced. Interestingly, although also some cases of cross-immunity have been reported, e.g. NukHFEG against the closely related lacticin 481, most lantibiotic producers have their own specific immunity mechanisms. It should be emphasized that due to the presence of immunity mechanisms, there is a potential risk that immunity is acquired through horizontal gene transfer where bacteria that are currently sensitive to specific lantibiotics gain resistance.

4. Lantibiotic biosynthesis enzymes

4.1 Structural peptides (LanA)

The LanA precursor peptide consist of an N-terminal leader peptide and a C-terminal core peptide (figure 1). The N-terminal leader peptide is generally between 23 and 59 amino acids long, and although it can contain serine and threonine residues it does not contain cysteine residues. The precise mechanism by which the leader peptide acts is not yet fully understood (2). However, it plays a significant role in targeting the peptide to the biosynthesis and transport proteins. Importantly, the lantibiotic remains inactive until the leader peptide is removed and thus provides intrinsic protection to the producer cell.

The leader peptides of the two main lantibiotic classes have their own characteristics. Class I leader peptides usually are about 25 amino acids long and rich in aspartate residues. Sequence alignment (2, 40) suggests a distinct motif, the FxLD motif, around positions -20 till -15. At position -2, there is usually a proline residue (figure 5). Class II leader peptides are rich in aspartate and glutamate residues and also contain a conserved motif, ELxxBx (B = V, L or I) at position -8 till -3 (2). This motif is usually followed by a double glycine motif, GG, GA, GS or GT, marking the proteolytic cleavage site that is recognized by the class II lantibiotic associated transporters (figure 5). Remarkably, the leader peptides of the two-component lantibiotics do not contain the ELxxBxx motif but do have the double glycine motif. In vitro studies on lacticin 481 suggest that the leader peptide plays a role in efficient modification of the core peptide, but it is not necessary for modification per se. When the core peptide of lacticin 481 was in vitro offered alone or in trans with the leader peptide, modification occurred although with a lesser efficiency (5, 41). Besides LctM, EpiD which is an oxidative decarboxylase providing a further modification of the lantibiotics, was able to modify substrates even in the absence of the leader peptide (42). These results seem to contradict a critical role of the leader peptide in recognition by the modification enzymes. Furthermore, leader peptides of class II bacteriocins that are not post-translationally modified also contain the characteristic double glycine motif in addition to an E(L/B)xxBx motif (2). Site-directed mutagenesis studies have shown that the double glycine motif is not essential for modification of the core peptide (5). However, the double glycine motif is important for proteolytic processing by the peptidase C39 domain of the bifunctional transporters (43, 44). On the other hand, some class II lantibiotics including

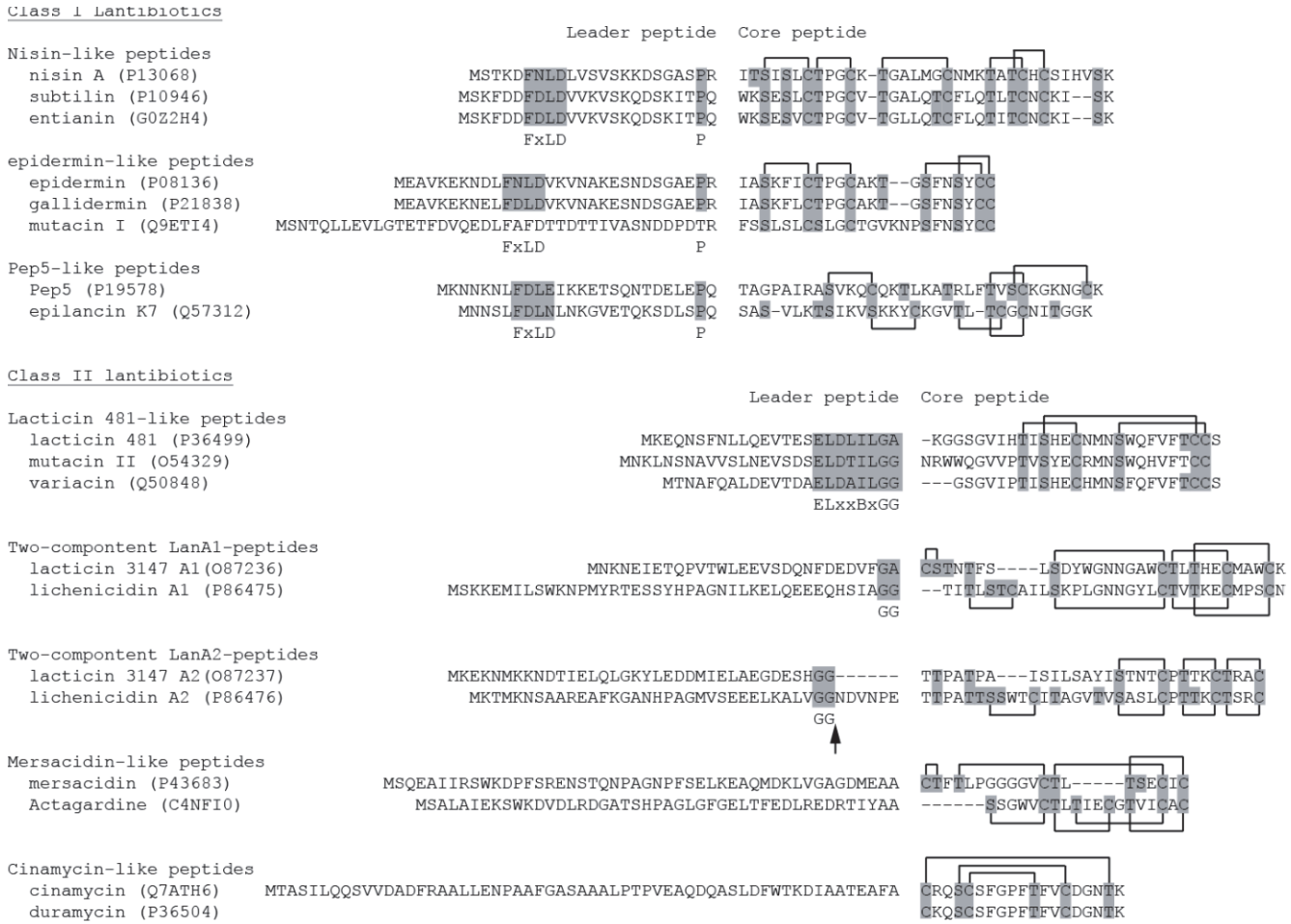

Figure 5: Alignment of leader and core peptides of subclasses within class I and class II lantibiotics. Class I leader peptides contain the conserved FxLD motif and a proline residue at position -2. In class II leader peptides the conserved double glycine cleavage site preceded in lacticin 481-like peptides by the ELxxBx motif. The modified residues of the core peptides are highlighted by grey boxes and the residues involved in (methyl)lanthionine bridges are connected by black lines. The black arrow underneath the lichenicidin A2 peptide indicates the cleavage site behind the double glycine motif leaving the 6 amino acids that require an extra proteolysis step to a mature peptide.

mersacidin-like and cinamycin-like peptides, contain relatively long leader peptides that do not comprise the ELxxBxGG motif. Taken together it could very well be that non-conserved amino acids and/or the structure of the leader peptides play a role in the recognition by the specific modification enzymes.

The C-terminal core peptide of the precursor peptide is post-translationally modified and after leader peptide removal the mature lantibiotic is released. Most lantibiotics are between 19-38 amino acids long. Class I lantibiotics are generally more elongated while class II peptides are more globular. Within these two classes, peptides can be further grouped based on their structure and similarity (45) (figure 5). For a complete list of lanthipeptides identified up to 2013 see the review by Dischinger et al. (45). Some distinct groups in class I lantibiotics are the nisin-, epidermin- and Pep5-like peptides. The nisin-like peptides include nisin A and its variants Q, U, Z, F as well as subtilin, entianin and others. These peptides have an overall elongated conformation with two amphipathic screw-shaped domains, one N-terminal domain formed by rings A, B and C and a second C-terminal domain formed by rings D and E. The two domains are connected by a flexible hinge region. The epidermin-like peptides include in addition to epidermin its natural variant gallidermin and the closely related mutacin peptides. This group has a conserved lanthionine ring at position 3 till 7 that is also found in the nisin-like group and that has been shown to play a role in activity (46, 47). Pep5-like peptides include Pep5, two variants of epilancin and epicidin 280. Pep5 contains eight positive charged residues rendering this peptide highly basic.

Since class II lantibiotics represent the largest group at present, they possess diverse structures. One of these subgroups are the lacticin 481-like peptides, containing among others lacticin 481, variacin, nukacin ISK-1 and mutacin II (48). The members share high sequence homology and are equipped with a similar ring formation pattern. They are all hydrophobic and bear no net charge at pH 7. The N-terminus of the mature peptide is usually linear while the C-terminus is globular due to the three overlapping (Me)Lan bridges. Mersacidin-like peptides are among the smaller lantibiotics with an average number of 20 amino acids and a compact globular structure. The cinnamycin-like group is composed of cinnamycin, duramycin, duramycin B and C and ancovenin. All cinnamycin-like lantibiotics are 19 amino acids long and produced by *Streptomyces spp.* They share a similar amino acid sequence and (Me)Lan ring

structure. Also characteristic for this group is the presence of a conserved lysinoalanine ring and, except for ancovenin, a hydroxyaspartate. In the group of the two-component peptides reside among others lacticin 3147, staphylococcin C55, plantaricin W, haloduracin and cytolysin. Except for cytolysin, the α-peptides (LanA1) resemble mersacidin while the β-peptides (LanA2) are long and screw-shaped (49). Structural data of lacticin 3147 (49) and haloduracin (50) in combination with bioinformatics studies suggest that the three rings of the α-peptides are conserved. The same holds for the last two C-terminal (Me)Lan rings in the β-peptides. In addition, the N-terminal methyllanthionine ring in HalA2 is conserved but not present in LtnA2.

4.2 (Methyl)lanthionine introducing modification enzymes

In class I lanthipeptides (methyl)lanthionine formation is performed by LanB and LanC. LanB enzymes are about 120 kDa in size and do not show any homology to other characterized proteins in the databases (51). A study from Garg et al. (52) on NisB, the dehydratase of nisin, showed that NisB requires ATP, glutamate and an unknown macromolecule to dehydrate threonine and serine residues in the nisin precursor peptide. A recent follow-up study (53) suggested that a nucleic acid in the form of a glutamyl-$tRNA^{Glu}$ is present constituting the unknown macromolecule. During dehydration, the hydroxyl groups of serine and threonine are converted to glutamyl esters. The glutamyl esters are then removed and as a result the carbon-carbon double bonds are formed in the dehydroalanine or dehydrobutarine residue (52) (figure 6A). A co-crystal structure of NisB and NisA (53) suggests that NisB forms a dimer with a bifurcated cleft-like structure in the center. In addition, crystallization and alanine substitution data show that the N-terminal region of NisB, about 700 residues, is responsible for the glutamylation and that a 300 residue C-terminal part of NisB is responsible for the glutamate elimination step (52, 53). Since LanB enzymes do not show any homology towards other known proteins, but are homologous among each other suggest that these enzymes catalyze the dehydration in the same manner. However, it has not yet been determined if all LanB enzymes dehydrate serine and threonine residues via glutamylation.

The cyclase enzymes (LanC) catalyze the thiol addition of a free cysteine to a dehydroalanine or dehydrobutyrine residues (figure 6B). Although LanC

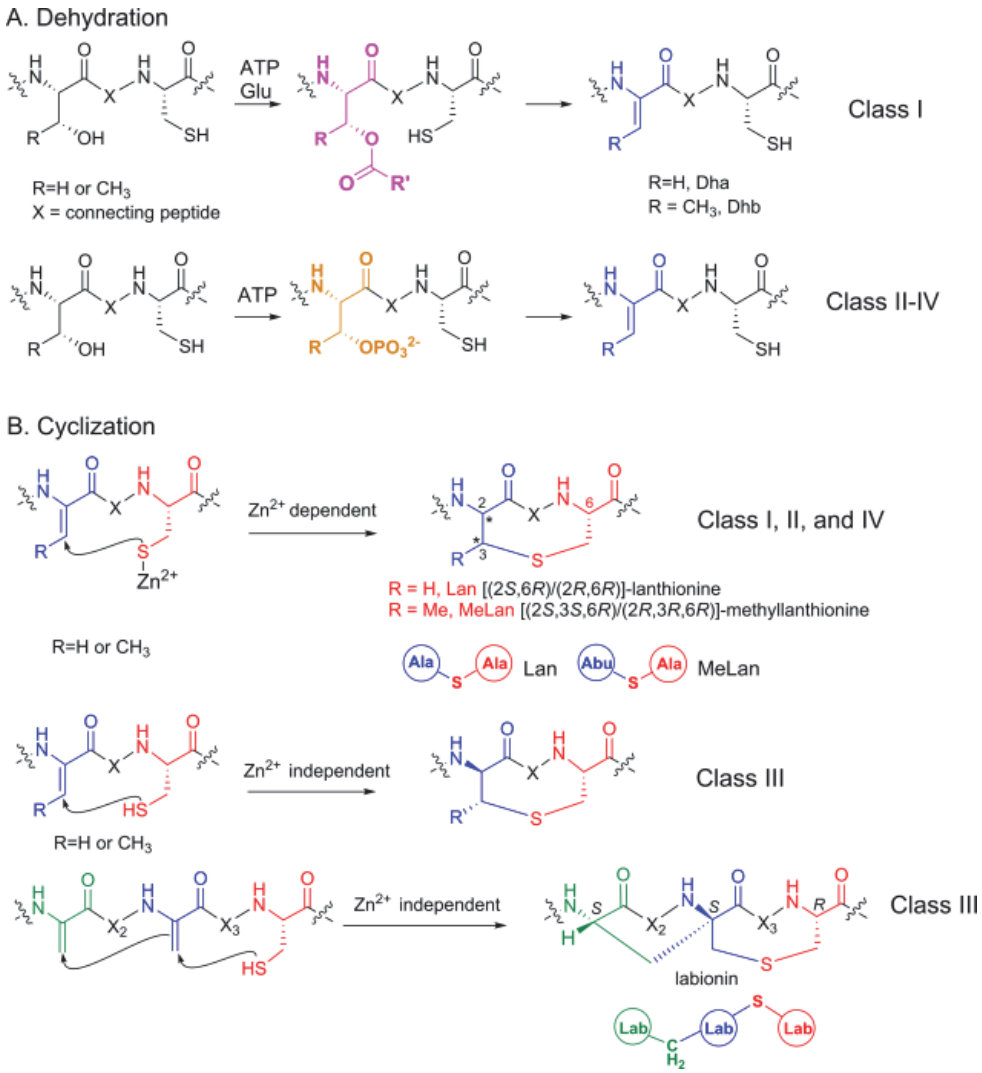

Figure 6: The dehydration (A) and cyclization (B) steps during the formation of (methyl)lanthionine and labionin residues. During dehydration the side chain hydroxyl group of Ser and Thr are activated to form dehydroamino acids (blue), dehydroalanine and dehydrobutyrine, respectively. Based on NisB studies it is believed class I enzymes dehydrated the amino acids via glutamylation (magenta) while the enzymes of class II-IV use phosphorylation (orange). In the cyclizations step in class I, II and IV the cysteine residue (red) is activated in a zinc dependent manner while for class III the cyclization step is zinc independent, via a currently unknown mechanism. Also class III enzymes can form labionin structures. These labionin structures are formed from one Cys (red) and two dehydroalanines (blue and green) connected via a carbon–carbon crosslink (8).
Dha, dehydroalanines; Dhb, dehydrobutyrine; Abu, 2-aminobutyric acid; Lab, labionin.

enzymes do not show sequence homology to known characterized peptides, they do show structural similarities with the β subunit of mammalian farnesyl

transferases (54). The crystal structure of NisC reveals two layers of α-barrels folded in a toroid and a connecting extended domain. The center of the toroid contains the zinc ion and the conserved zinc-ligands, His331, Cys284 and Cys330. This active site of LanC enzymes, with a Zn^{2+} ion, activates the thiol in the cysteine. The residues His212, Arg280, Asp141 and Tyr285 are also conserved throughout the LanC proteins. These four residues are thought to play a role in deprotonation of the cysteine residue and the protonation of the enolate. However, mutagenesis studies on Arg280 and Tyr285 of NisC (15), and corresponding residues in SpaC (55), show that these residues are not essential for cyclization. In addition, residues His212 and Asp141 are critical for correct cyclization of nisin, the His212 residue is even conserved in the LanC-like cyclase domains of LanM proteins supporting the importance of the amino acid. A study by Li et al. (54) showed that NisC requires the leader peptide for the cyclization reaction, since a leader-less dehydrated prenisin is not converted to mature nisin. The study also showed that adenosine triphosphate (ATP) is not required for the cyclization reaction. Furthermore, dehydrated prenisin was converted into nisin by SpaC, the cyclase enzyme involved in the biosynthesis of subtilin. This suggest that dehydration and cyclization in class I lantibiotics occur independently.

Class II lantibiotics contain one multifunctional modification enzyme, LanM, that performs both the dehydration and cyclization reaction. LanM enzymes are about 110-120 kDa in size and contain two domains. The N-terminal dehydratase domain does not show any significant sequence identity with LanB (56), but only shows sequence homology to other LanM enzymes (51). It is believed that class II LanM enzymes first phosphorylate the serine or threonine residues, after which the phosphate is removed to generate a dehydroalanine or dehydrobutarine, respectively (57) (figure 6A). The C-terminal part of LanM shows homology to LanC enzymes including the conserved zinc-ligands. Therefore it is believed that the cyclization in class II lantibiotics is catalyzed in the same zinc dependent manner as in class I cyclization (figure 6B).

4.3 Other post-translational modification enzymes

Lantibiotics can undergo further post-translational modifications. Fifteen different post-translational modifications have been reported (58). It is believed

that these extra modifications increase the peptide stability and activity. One of these extra modifications is the formation of an *S*-aminovinyl-D-cysteine (AviCys) amino acid. The enzyme responsible for this oxidative decarboxylation at the C-terminus of the lantibiotic is generically termed LanD. The formation of such an AviCys residue protects the peptide from carboxypeptidases (59, 60). A extensively studied LanD protein is EpiD from *Staphylococcus epidermidis*. EpiD belongs to the homooligomeric flavin-containing Cys decarboxylase (HFCD) family (22). The consensus sequence of the C-terminal amino acids for the recognition by EpiD is [V/I/L/F/W/Y/(M)]-[A/S/V/T/C/(I/L)]-C (42). Another example is MrsD that forms the *S*-aminovinyl-3-methyl-D-cysteine (AviMeCys) amino acid in its substrate mersacidin. MrsD is a flavin adenine dinucleotide (FAD)-containing enzyme. The crystal structures of EpiD (61) and MrsD (62) revealed a conserved Rossman fold typically found in flavodoxin binding proteins. GdmD which belongs to the gallidermin biosynthetic gene cluster of *Staphylococcus gallinarum* was shown to modify also other lanthipeptides than gallidermin, namely a nisin variant when co-expressed in *Lactococcus lactis* (63).

Spontaneously hydrolysis of N-terminally exposed dehydroalanine and dehydrobutyrine residues can occur. These residues become exposed after leader peptide removal and are subsequently hydrolyzed to 2-oxopropionyl (OPr) and 2-oxobutyryl (OBu) groups (64). Moreover, LanJ can convert L-serine to D-alanine. Until now only two lanthipeptides contain D-alanine residues, lactocin S and both peptides of the two-component lantibiotic lacticin 3147, i.e., LtnA1 contains one and LtnA2 holds two D-alanine residues. The LtnJ enzyme involved in this process shows homology to zinc-dependent alcohol dehydrogenases and NAD(P)H-dependent quinone oxidoreductases of the zinc-containing alcohol dehydrogenase superfamily (65). The first step in generating a D-alanine is the dehydration of a serine to form a Dha residue by LtnM1 or LtnM2 (65). Next, the stereospecific hydrogenation of Dha occurs resulting in the formation of D-alanine (66).

4.4 Lantibiotic transport and processing

Lantibiotics are excreted from the cell by dedicated ATP-binding cassette (ABC) transporters (figure 7). ABC transporters associated with class I lantibiotics, named NisT-type transporters, are about 550 to 600 residues in size.

These transporters contain 3 domains, an N-terminal hydrophobic domain, a six-helix membrane spanning permease domain and a C-terminal ATP-binding domain. For both nisin and subtilin a multimeric complex between the transporter and the modification enzymes can be formed (67, 68). However, complex formation is not a requirement for transport as without NisC, dehydrated NisA could be secreted. In addition, NisT alone is able to export unmodified nisin or non-related peptides fused behind the nisin leader peptide (69). These findings show that NisT is equipped with a low substrate specificity.

To obtain the mature and active lantibiotic, the N-terminal leader peptide needs to be removed. In class I lantibiotics a dedicated subtilisin-like serine protease, generally termed LanP, removes the leader peptide commonly at the extracellular face of the membrane. These LanP proteases can vary in size depending if they contain an N-terminal signal sequence and a C-terminal cell wall anchor sequence (22). Serine proteases contain conserved residues in their catalytic site. This concerns an aspartate, histidine and serine residues as well as a conserved asparagine residue at the oxyanion hole. Generally LanP is a extracellular protease and either membrane anchored, e.g. NisP, or soluble extracellular, like EpiP. However, PepP and ElkP are two examples of serine proteinases that lack a signal sequence and that have been suggested to localize in the cytoplasm.

Transporters associated with class II lantibiotics belong to the family of ABC transporter maturation and secretion (AMS) proteins, also known as SunT-type transporters, named after the characterized member SunT (72). These transporters are about 700 amino acids long and contain an N-terminal peptidase domain belonging to the peptidase C39 family, a central transmembrane domain and a C-terminal ATP-binding domain (figure 7). LcnC is a bacteriocin transporter that belongs to the SunT-type transporters, and for which a cytosolic localization of the N-terminal protease domain was demonstrated (73). A similar result was obtained for NukT, the lantibiotic nukacin ISK-1 transporter (74). Six transmembrane helices in the central permease domain of the transporter were suggested, and typically in such ABC transporters, this domain homodimerizes with another copy of the ABC transporter. Some of these proteins consist of two individual subunits that heterodimerize. The ATP binding domain and the peptidase domain seem to function cooperatively, as mutations in either domain

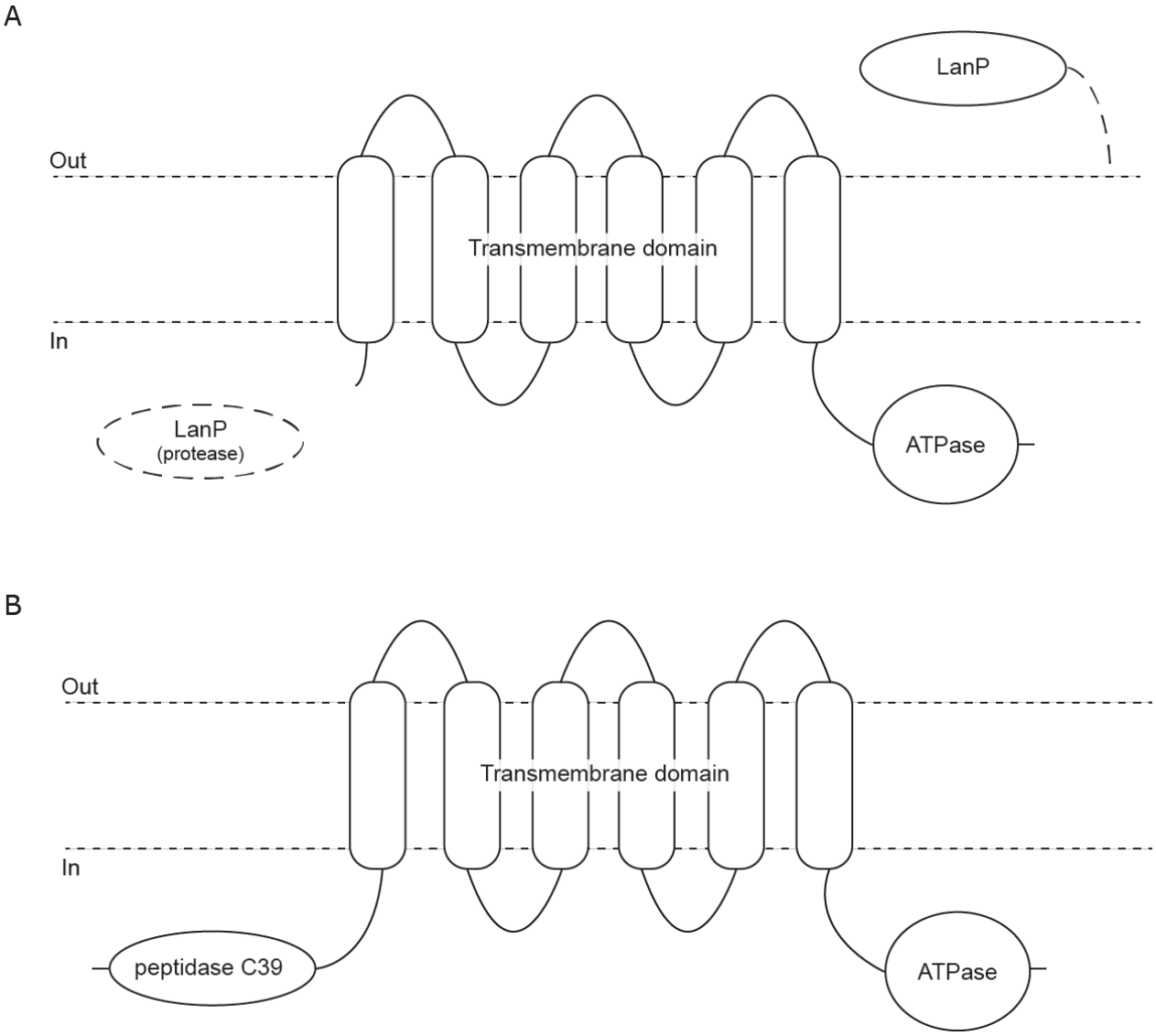

Figure 7: Schematic representation of lantibiotic transport and processing enzymes. With a separate ABC-transporter and serine protease for class I lantibiotics (A) and a multifunctional ABC transporter maturation and secretion (AMS) protein for class II lantibiotics (B).

of the NukT transporter abolish the capability to transport and cleave the leader peptide (74). The crystal structure of ComA, an AMS protein (4) showed that the peptidase domain corresponds to the structure of papain-like cysteine proteases. These cysteine proteases contain conserved glutamine, cysteine, histidine and aspartate/asparagine residues that when mutated cause the loss of the peptidase activity (44, 75, 76). Many of these transporters are involved in the export of bacteriocins (non-lantibiotic and lantibiotic), and the corresponding substrates are processed at the so called double glycine site (GG/GA/GS/GT) at the C-terminus of the leader peptide. The conserved cysteine and histidine residues are

part of the catalytic site, while the conserved glutamine is believed to be associated with an oxyanion hole (76).

In addition to the double glycine motif, the α-helical structure of the leader peptide seems to be important for recognition by the peptidase C39 domain (44). In addition it was proposed that the peptidase domain contains a hydrophobic patch that interacts with the hydrophobic surface of the α-helix of the leader peptide (3). The peptides of cytolysin require a second proteolytic step by an extracellular serine protease, CylA. This protease removes a six amino acid long sequence from the N-terminus of, $CylL_L$ and $CylL_S$, to yield the active cytolysin components (77). The β-peptides of plantaricin W, haloduracin and lichenicidin also seems to require an extra round of proteolysis. However, the responsible proteases have not yet been identified (50, 78, 79).

5. Lantibiotic mode of action

Many lantibiotics are antimicrobial agents, but their exact mode of action has been studied only for a few lantibiotics. It is believed that the antimicrobial activity is mainly based on the inhibition of cell wall biosynthesis, disruption of membrane integrity by forming transmembrane pores, or a combination of both (10). Lantibiotics act mostly against Gram-positive bacteria. Gram-negative bacteria are usually not affected since they have an outer membrane that forms a barrier to the entering lantibiotic.

The mode of action of nisin has extensively been studied and two mechanisms appear to be operational, namely pore formation and the inhibition of cell wall biosynthesis. Nisin uses lipid II, an amphipathic peptidoglycan precursor of cell wall biosynthesis as a docking molecule. Clinically used antibiotics, like vancomycin, also target lipid II and bind the D-ala-D-alanyl group of lipid II. Lantibiotics bind a different site. For example, the N-terminal A and B rings of nisin form a cage that binds the pyrophosphate moiety of lipid II (11, 80). The A and B rings are conserved in nisin- and epidermin-like peptides (figure 5) and thus the same binding motif can be found in these lantibiotics (81, 82). Binding of the lantibiotic to lipid II causes inhibition of the cell wall biosynthesis. A recent study (82) showed that nisin and mutacin 1140 disrupt the functional localization of the lipid II. Following lipid II binding, complexes of nisin and lipid II cluster together and the C-terminus of nisin inserts into the membrane to form pores. The pores are 2-2.5 nm in diameter and consist of

eight nisin and four lipid II molecules (83, 84). In addition, Nisin and subtilin also inhibit spore outgrowth. Pep5 also acts by pore formation, but little is known about the membrane interaction (85).

Class II lacticin 481-like peptides are closely related in sequence and predicted structure. Moreover, they share a similar self-immunity mechanism that removes the peptide from the membrane. Streptococcin A-FF22 has been shown to form unstable membrane pores thereby disrupting the membrane potential and impairing ATP production (86). Therefore, it is believed that lacticin 481-like peptides target the membrane by forming pores (48). Indeed, lacticin 481 (87) and nukacin ISK-1 (88) interact with artificial lipid monolayers, while mutacin II (89) causes a depletion of the intracellular ATP pool. However, the exact mechanism of pore formation by these lantibiotics has not yet been determined.

Lipid II is also the target for mersacidin, that inhibits peptidoglycan biosynthesis at the level of transglycosylation (90). Mersacidin does, however, not form pores in the membrane. The C-ring and the glutamic acid in this ring are essential for lipid II binding and the peptide changes its conformation upon binding (91, 92). The ring is conserved also in the lacticin 481-like peptides and the α-peptides of the two-component peptides, suggesting a similar mode of action for these lantibiotics.

Two-component lantibiotics also use a dual mode of action. The individual peptides display no or only a weak antimicrobial activity, but strong activity is observed when both peptides are present. Except for cytolysin, the α-peptides of the two-component lantibiotics usually resemble the structure of mersacidin. Since the three C-terminal rings have the same topology as in mersacidin it is assumed that these peptides target lipid II. The β-peptides have a more elongated structure, and it has been suggested that they are involved in the pore forming activity. With the two-component lantibiotic lacticin 3147, both peptides work synergistically in a ratio of 1:1 (93, 94). LtnA1 forms a complex with lipid II thereby inhibiting cell wall biosynthesis, while LtnA2 then binds to this complex and forms a pore in the membrane (95).

Other activities reported for lantibiotics include a cytolytic effect on erythrocytes and polymorphonuclear leukocytes by cytolysin (77). Among the group of cinnamycin-like peptides, some inhibit phospholipase A2 by sequestering the phosphatidylethanolamine (PE) substrate (96).

6. Engineering and applications of lantibiotics

Lantibiotics are gene encoded and ribosomally synthesized and therefore they can be more readily engineered than non-ribosomal or polyketide antibiotics (97). Lantibiotic engineering has mainly been employed for structure-function studies and to identify essential amino acid residues. Nisin is one of the best studied lantibiotics because of a very efficient expression system. Expression systems have also been created for other lantibiotics like subtilin, Pep5, epidermin, gallidermin, mutacin II, lacticin 481, lacticin 3147 and mersacidin (17, 98-102). Most of these systems use homologous or closely related Gram-positive bacteria as a host strain. However, lantibiotics are naturally expressed by and demonstrate activity towards Gram-positive bacteria, and thus there is an inherent risk that the engineered lanthipeptide is active against the production host. As an alternative, expression in the Gram-negative *Escherichia coli* has been attempted where the peptide is made intracellular in its inactive, leader peptide containing form. Examples of lanthipeptides that are heterologous expressed are nukacin ISK-1 (103), nisin, prochlorosins and haloduracin (104), cinnamycin (105) and lichenicidin (106). By genome-mining, lantibiotic and associating genes are readily identified, and vast numbers of such peptides appear to be produced by bacteria. However, expressing such lantibiotics by the natural host is complex as often they emerge from non-cultivatable bacteria. Therefore, the heterologous expression in *E. coli* may be employed to increase concentrations and produce such putative lantibiotics.

Although lantibiotics contain their own specific export systems, the possibility to employ the general Sec or Tat export systems to export lantibiotics has been investigated. Kuipers et al. (107) showed that when the Tat or Sec signal sequence is fused to the nisin leader peptide in a strain lacking NisT, the dehydrated nisin was secreted. However, a fully modified nisin also containing (methyl)lanthionine-bridges could not be transported.

Engineering can also be used to increase the activity of lantibiotics or make them more suitable for applications. For example, nisin variants with higher activity and enhanced solubility were created (108). In addition, mutagenesis of ring A of nisin resulted in two peptides with higher antimicrobial activity (109). For mersacidin, new variants have been patented (110, 111). In addition, introduction of a commercial protease recognition site replacing the native cleavage site (40, 104), has been shown to be advantageous for the

production of an inactive lantibiotic that post-production can be activated through the use of a specific protease.

The lantibiotic biosynthesis enzymes can also be used to engineer peptides that normally do not contain such modifications. This might provide native peptides with an altered biological activity, different specificity or increased resistance to proteolysis. LtnM2T (112) of lacticin 3147 and NisBTC (113) of nisin have been shown to be able to introduce the characteristic modifications into non-lanthionine peptides that were fused behind the appropriate leader peptides. Also, Kluskens at al. (114) demonstrated that NisBTC successfully dehydrates and secretes non-lantibiotic peptides like vasopressin, angiotensin, and enkephalin. These findings represent a promising application of the lantibiotic modifying enzymes, and suggest that these enzymes are equipped with a low substrate specificity. This could be exploited for the bioengineering of a wide range of peptides.

Lantibiotic regulation genes are widely used to control gene expression. Well-known examples are the nisin-controlled gene expression (NICE) and subtilin-regulated gene expression (SURE) systems (26, 27). These systems are broadly used to express non-lantibiotic related genes in *L. lactis* and *B. subtilis*. On the other hand they can also be used to express biosynthetic genes of lantibiotics for the heterologous production of lanthipeptides or analysis of lantibiotic biosynthetic enzymes. Examples are the production of nukacin ISK-1 in *L. lactis* using the NICE system (115) and the study of enzyme complexes involved in the biosynthesis of lacticin 3147 and nisin (112).

A further application is the use of lantibiotic synthetase enzymes in vitro. This has the advantage to introduce (methyl)lanthionine-rings in peptides in the absence of the cellular quality control mechanisms. In addition, it overcomes the potential secretion problem and adverse effects of the bioactivity of the lantibiotic towards the producing host strain. The first in vitro application was the lantibiotic synthetase of lacticin 481 (LctM) (116). Both LctM and lctA where separately and heterologously expressed in *E. coli*, purified and combined in vitro. The system was used to test the substrate specificity of LctM. Also, HalM (50), NisB (52) and NisC (54) have been used in vitro.

An example of successful commercial application of a lantibiotic is nisin. It is already for over 50 years used as a food preservative without significant development of microbial resistance. Lantibiotics are also interesting peptides to

be used as antimicrobials because of the target (lipid II) and the low probability to generate resistance. Lantibiotics have shown a broad activity towards Gram-positive pathogens, for example mersacidin exhibits activity against methicillin-resistant *Staphylococcus aureus* in a murine model (117) and against vancomycin resistant enterococci (90). In addition, the presence of a (methyl)lanthionine residue is believed to increase the biostability and resistance against proteolytic degradation (118, 119) making them excellent candidates for the creation of novel antibiotic structures. A limitation, however, is that these lantibiotics are not active against Gram-negative bacteria limiting the use as narrow spectrum antibiotics. In addition, most lantibiotics are not stable at a neutral or basic pH, resulting in oxidation of the (methyl)lanthionine bridges and loss of activity (120, 121). This potentially interferes with a medical application of lanthipeptides. An example of a lanthipeptide used for medical applications is microbisporicin that is active against nosocomial infections (122). An analog of actagardine can be employed for the treatment of *Clostridium difficile* infections (123). Duramycin has potential for the treatment of cystic fibrosis since it increases the chloride secretion in lung epithelium (124, 125). Mutacin 1140 is in preclinical development as treatment against Gram-positive bacterial infections (126). Nisin and nisin variants show anti-tumor activity in head and neck cancer (130). Since cytolysin can also act as a virulence factor, lantibiotics could also play a role in other therapeutic applications. Because of issues with penetration and solubility, most current medical applications concern surface application that require formulations such as in skin creams.

Lantibiotics not only have potential in human healthcare but may also be used in veterinary healthcare (127). Another application is in molecular imaging. Zhao et al. (128) described the possibility to use duramycin as a molecular imaging probe, since it exhibits a high affinity for phosphatidylethanolamine an abundant phospholipid in cell membranes of mammalian cells.

7. concluding remarks

Lanthipeptides constitute a large group of ribosomally synthesized and post–translationally modified peptides. Lately, research on these lanthipeptides has accelerated at a high speed. More and more lanthipeptide associated genes have been discovered through metagenome searches and new classes, e.g. class IV, and posttranslational enzymes have been identified. The group of lantibiotics

specify antimicrobial activity, they are structurally diverse and modified by separate dehydratase and cyclase enzymes (class I) or multifunctional enzymes (class II). Although many of the intimate details on lantibiotic biosynthesis have been elucidated, more insight is needed on the precise mechanisms of modification, substrate recognition, and the function of the actual leader peptides. Bioengineering of known and novel lanthipeptides using in vitro and in vivo techniques exploiting the promiscuity of the lantibiotic biosynthesis enzymes is an emerging field that will allow the development of novel agents that can be applied as therapeutics.

8. Scope of thesis

The research described in this thesis focuses on the development of an expression system for lanthipeptides that belong to class II. The envisioned production system contains a known lantibiotic modification enzyme and possibly the associated transporter. The first half of the thesis embarks on the development of a class II lanthipeptide production system in *Lactococcus lactis* employing the modification enzymes LtnM1 or LtnM2 and the transporter LtnT of lacticin 3147. While the second half will investigate *Escherichia coli* as a heterologous host for such a production system.

Chapter 1 provides an overview of our current understanding of the biosynthesis of the diverse classes of lanthipeptides, their activities and mode of action as well as their potential for applications.

Chapter 2 investigates the function of LtnT. The catalytic cysteine of the N-terminal peptidase C39 domain was mutated and the domain was deleted, after which the ability of these variants to transport the associated substrates was evaluated. The data suggest that LtnT requires an active peptidase C39 domain for transport.

Chapter 3 examines whether the modification enzymes, LtnM1 and LtnM2, in combination with LtnT modify, process and secrete their cognate substrates LtnA1 and LtnA2, respectively. Modified and processed peptides were detected and an active lacticin 3147 could be produced.

Chapter 4 further evaluates the substrate specificity of the LtnM1T and LtnM2T systems. Variants of LtnA1 and LtnA2 were created including a swap of the respective leader peptides, an all cysteine to alanine mutation to prevent thioether bridge formation and the introduction of a factor Xa protease site in the

leader peptide cleavage region of LtnA1. This work revealed a high specificity of the LtnM1 and LtnM2 enzymes towards their cognate substrates, which limits a more general application of these enzymes.

Chapter 5 describes the construction of a production system in *E.* coli using the modification enzymes of lacticin 3147 (LtnM2) and mutacin II (MutM). Both enzymes were found to be active in *E. coli* producing fully dehydrated MutA and LtnA2, respectively. Also, the modification enzymes were found to be capable of dehydrating a diverse range of peptides.

In chapter 6, a putative lanthionine synthetase and its associated structural peptide from *Streptococcus pneumoniae* Spain 23F-1 were expressed in *E. coli*. LanM-SPN23F was shown to be active, producing a 7 till 8 times dehydrated novel lanthipeptide. In addition, GdmD (LanD) was co-expressed and found to decarboxylate the LanA-SPN23F peptide.

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Chapter 2

The role of the cytosolic peptidase domain of the lantibiotic ABC transporter LtnT in the secretion of lacticin 3147

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Abstract

LtnT of *Lactococcus lactis* subsp. *lactis* DPC3147 belongs to the ABC transporter maturation and secretion (AMS) protein family. It mediates the secretion of the lanthipeptides LtnA1 and LtnA2, which together constitute the two-component class II lantibiotic lacticin 3147. In addition, LtnT processes the leader peptide by means of an intracellular N-terminal peptidase C39 domain. In contrast, class I lantibiotics depend on a specific extracellular proteinase for processing. To investigate the importance of this domain in the transport mechanism of LtnT, the catalytic cysteine was mutated and the N-terminal peptidase domain was deleted. In both cases, this completely abolished the production of LtnA1 or LntA2. These data suggest that LtnT requires an active peptidase C39 domain for transport of its cognate substrates.

Introduction

Lantibiotics, lanthionine-containing antibiotic peptides, harbor (methyl)lanthionine, dehydroalanine and/or dehydrobutarine residues that are post translationally introduced into the peptides. In class I lantibiotics (e.g. nisin) the modifications are introduced by two enzymes, LanB a dehydratase and LanC a cyclase. In class II lantibiotics (e.g. nukacin ISK-1 or lacticin 3147) the modifications are done by a bifunctional modification enzyme, LanM. The lantibiotic precursor peptide LanA consist of a N-terminal leader peptide and a C-terminal core peptide. Only the latter is post-translationally modified by the before mentioned enzymes. In order to obtain the mature and active peptide, the N-terminal leader peptide needs to be removed. In class I lantibiotics, an extracellular serine protease, generally termed LanP, removes the leader peptide after transport. In class II lantibiotics, the leader is removed concomitant with, or prior to transport by a dedicated bifunctional ATP-Binding Cassette (ABC) transporter, LanT (1).

Transporters associated with class II lantibiotics are ABC transporter maturation and secretion (AMS) proteins, also known as SunT-type transporters, named after the characterized member SunT (2). These transporters consist of approximately 700 amino acids and contain a N-terminal peptidase domain belonging to the peptidase C39 family, a central transmembrane domain and a C-terminal ATP-binding domain (figure 1A). This peptidase domain is localized at the cytoplasmic side of the membrane (3, 4). Therefore, it is assumed that there is an even number, namely four or six, transmembrane helices in the central domain of the transporter. In addition, the ATP binding and peptidase domain appear to function cooperatively (4). Mutations in either domain of the NukT transporter abolishes the capability to transport the lanthipeptide and to cleave the leader peptide. The crystal structure of ComA, an AMS protein, has been solved (5) showing that the peptidase domain has a similar structure as papain-like cysteine proteases. These cysteine proteases contain a conserved cysteine residue and several other conserved residues that when mutated cause the loss of peptidase activity (6-8). The substrates of the peptidase domain containing ABC transporters are usually bacteriocins (non-lantibiotic and lantibiotic) that are processed at a so-called double glycine site (GG/GA/GS/GT) at the C-terminus of

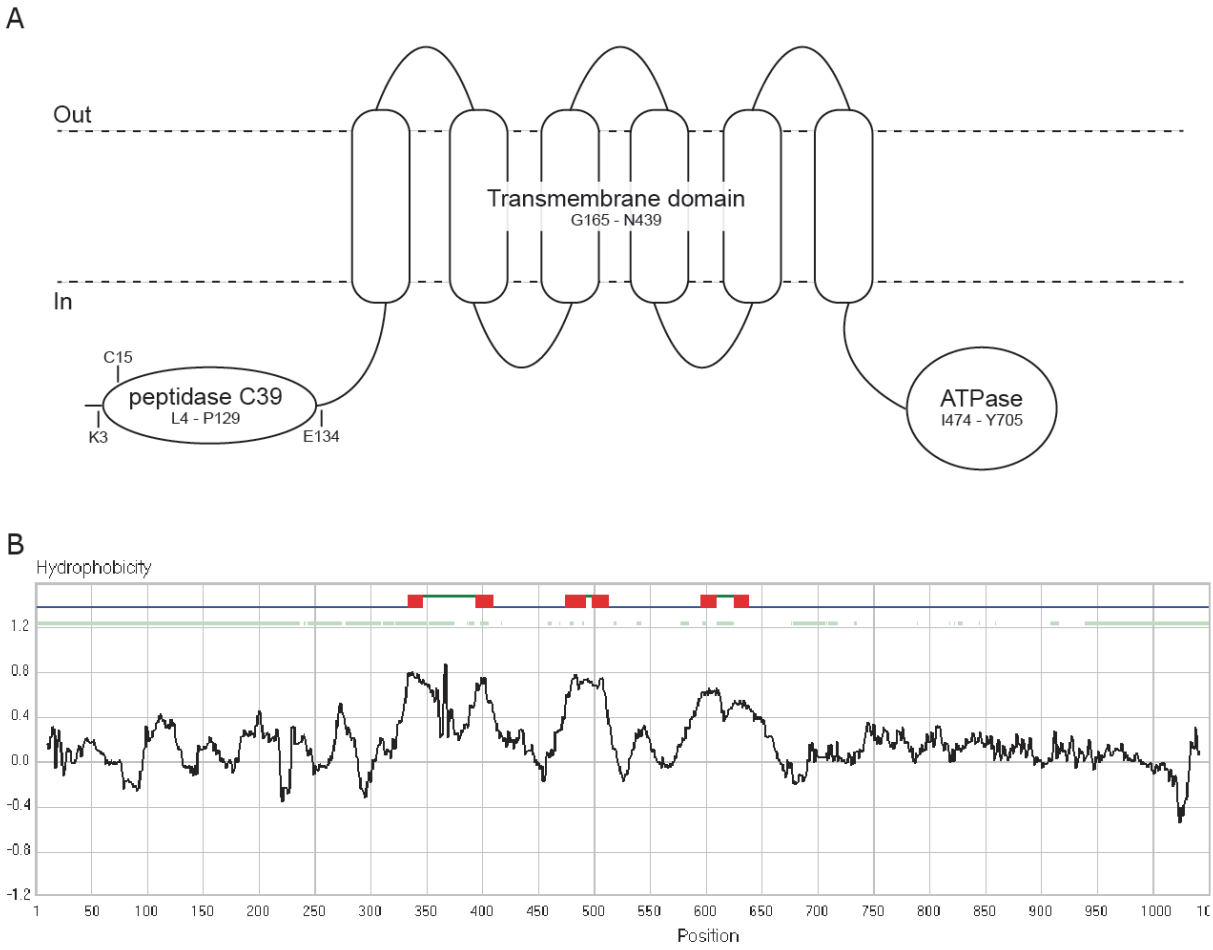

Figure 1: A) schematic representation of the three domains of the lacticin 3147 transporter LtnT. C15 indicates the position of the catalytic active cysteine, that was substituted for an alanine in LtnT-C15A. The N-terminus including residues 3 to 134 was removed to obtain the truncated trLtnT mutant. B) Hydrophobicity plot showing the six transmembrane domains for proteins belonging to the ABC transporter maturation and secretion (AMS) protein family which includes LtnT. The hydrophobicity plot was generated on 120 proteins homologous to LtnT using MemGen 4.2.0 (14).

the leader peptide. In addition to the double glycine motif, the α-helical structure of the leader peptide seems to be important for the recognition by the peptidase C39 domain (7). Furthermore it was proposed that the peptidase domain contains a hydrophobic patch that interacts with the hydrophobic surface of the α-helix of the leader peptide (9). The catalytic site of the peptidase domain contains a conserved cysteine and histidine residue, whereas a conserved glutamine is believed to be associated with the oxyanion hole typically found in peptidases that belong to the cysteine protease family (8).

The transporter of the two-component lantibiotic lacticin 3147, LtnT, functions as a bifunctional transporter catalyzing both the processing and transport of these lantibiotics. Although there are some reports on the structure and export mechanism of SunT-type transporters (2), little is known about the specificity of the lacticin 3147 transporter and the role of its peptidase domain. Here LtnT variants with a mutation and deletion in the peptidase domain were analyzed for their ability to process and transport substrate peptides to assess the role of the peptidase C39 domain in the transport mechanism.

Material and Methods

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in table 1. *Lactococcus lactis* NZ9000 was used as a host for the plasmids. All strains were grown at 30°C in Bacto M17 broth (Becton Dickinson Difco) supplemented with 0.5% glucose. Where needed chloramphenicol (5 µg/mL), erythromycin (5 µg/mL) or both antibiotics (3 µg/mL) were added to the medium.

Table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
Lactococcus lactis NZ9000	MG1363 derivative; pepN::nisRK+	(17)
Plasmids		
pIL253	Em^r	(18)
pILPltnT-2	pIL253 derived; *ltnT-ltnM2* cloned behind the *Pnis* promoter, disruption of ltnM2 by BglII digestion; Em^r	(11)
pILPltnT-2-his	pILPltnT-2 derived; containing His-tag at C-terminus of LtnT; Em^r	This study
pILPltnT-C15A	pILPltnT-2 derived; mutation of active cysteine at position 15 to alanine; Em^r	This study
pILPltnT-C15A-his	pILPltnT-C15A derived; containing His-tag at C-terminus of LtnT-C15A; Em^r	This study
pILPtrltnT-b	pILPltnT-2 derived; disruption of C39 peptidase domain by removing Lys3-Glu134 of LtnT; Em^r	This study
pILPtrltnT-b-his	pILPtrltnT-b derived; containing His-tag at C-terminus of trLtnT; Em^r	This study
pNZ8048	Cm^r	(17)
pA24	pNZ8048 derived; *ltnA2* cloned behind the *Pnis* promoter; Cm^r	(11)
pA13	pNZ8048 derived; *ltnA1* cloned behind the *Pnis* promoter; Cm^r	Kind gift from Lanthio Pharma

Em^r erythromycin resistance marker
Cm^r chloramphenicol resistance marker

Plasmid construction. Plasmid construction was carried out using standard procedures (10). Phusion High-Fidelity DNA Polymerase for PCR, restriction and DNA modifying enzymes were all purchased from Thermo Scientific. All sequencing analysis were performed at Macrogen Europe. PCR primers used to create the below described constructs can be found in table 2.

Table 2: Primers used in this study

Primer name	Primers sequence (5'-3')	Characteristics
045-fw	CTTTAAACAAGCTAGCAGAACGCCC	Amplification of LtnT-C15A fragment one, NheI restriction site
046-rv	CAGCAAAGTCCAGCTTCAGTTTGTG	Amplification of LtnT-C15A fragment one, C15A mutation
047-fw	ACAAACTGAAGCTGGACTTTGCTG	Amplification of LtnT-C15A fragment two, C15A mutation
048-rv	CTGTCGCTGCAGTGACATAGTTATTAG	Amplification of LtnT-C15A fragment two, PstI restriction site
159-fw	[Phos]CTTCATCCGGGGTGAGTG	Primer to remove Lys3-Glu134 of LtnT
160-rw	AAATTTAAAAAAAATACTGATAATATTATAAACAC	Primer to remove Lys3-Glu134 of LtnT
153-fw	CATCACCACTAATTTATTATTTTATAAATAAAATAC	Primer to introduce His-tag
154-rv	[Phos]ATGGTGATGCTTAGAAGTATATATTTTC	Primer to introduce His-tag

ATGC sequence containing restriction enzyme site, C15A mutation in LtnT or His-tag DNA sequence.

The pIL253 derived plasmids are based on pILPltnT-2 (11), which was a kind gift from Lanthio Pharma. This plasmid was used to create the following constructs. To generate a mutant of LtnT with a mutation at its presumed catalytic cysteine, plasmid pILPltnT-C15A was generated using overlap extension PCR. In de first step two products were generated, from pILPltnT-2, both containing the C15A mutation in LtnT and with an overlapping sequence of 24 bp. In de second step the overlapping products were annealed and extended with the outer primers to yield a single product. Subsequently, the DNA fragment was treated with NheI and PstI restriction enzymes followed by ligation into the NheI/PstI site of pILPltnT-2. The sequence of the resulting plasmid was analyzed for the presence of the C15A mutation. Mutagenesis PCR was also executed on pILPltnT-2 to delete residue Lys3-Glu134 of LtnT which correspond to the peptidase domain. This yielded pILPtrltnT-b.

LtnT, LtnT-C15A and trLtnT were fused to a C-terminal hexa histidine-tag at the C-terminus of LtnT using mutagenesis PCR. The corresponding transporter variants were cloned into respective pILP-plasmids yielding, pILPltnT-2-his, pILPltnT-C15A-his and pILPtrltnT-b-his.

The pNZ8048 derived plasmids (table 1), pA24 (11) and pA13, used in this study were a kind gift of Lanthio Pharma. After molecular cloning, *L. lactis* NZ9000 was transformed with the indicated pNZ8048 and pIL253 derived plasmids by electrotransformation (12) using a Bio-Rad gene pulser.

Expression of the His-tagged lacticin 3147 transporter. *L. lactis* NZ9000 cells were transformed with pILPltnT-2-His, pILPltnT-C15A-His or pILPtrltnT-b-His and grown overnight in GM17. Fresh GM17 was inoculated 1:60 with an overnight culture of the expression strain, incubated at 30°C till an optical density at 600 nm (OD_{600}) of 0.4-0.5 at which point expression was induced with nisin (Sigma-Aldrich) at a concentration of 1 ng/mL. After four hours of induction, cells were harvested by centrifugation at 16,100 x g for 7 minutes. The cell pellet was lysed by resuspension in 4 mg/mL Lysozyme (Roche) in MilliQ water and incubation for 30 minutes at 55°C. SDS-PAGE, Coomassie brilliant blue staining and immunoblotting were used to detect the LtnT transporters. Western blotting using an anti-His antibody (5 PRIME) was done according to standard protocols.

MALDI-TOF mass spectrometry. Minimal medium was prepared as described by Rink et. al. (13) with the vitamin mix replaced by BME Vitamins (Sigma-Aldrich). Minimal medium was inoculated 1:50 with an overnight culture of an expression strain grown in GM17. After three hours at 30°C, expression was induced by adding 1 ng/mL nisin (Sigma-Aldrich), and growth was continued for 24 hours. Next cells were removed from the culture by centrifugation (20 min., 3724 x g). A sample of 1 µL of the supernatant was applied to the MALDI-TOF target, dried and washed with a drop of MilliQ water. Subsequently, 1 µL of matrix, 5 mg/mL α-Cyano-4-hydroxycinnamic acid (Sigma-Aldrich) dissolved in 50% Acetonitrile and 0,1% trifluoroacetic acid (TFA), was added to the sample and dried.

In addition, peptides were precipitated from the culture supernatant by addition of 10% trichloroacetic acid (TCA). After 30 minutes on ice the sample

was spun for 30 minutes at 17,000 x g. The pellet was washed with acetone, centrifuged (15 min. at 17,000 x g) and dried (5 min. at 42°C). The TCA precipitated pellet was dissolved in 10 µL MilliQ water of which 1 µL was applied to the MALDI-TOF target, and treated as described above. Spectra were recorded with a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems) and analyzed with Data Explorer TM 4.0.0.0 (Applied Biosystems). An external calibration was used to correct the resulting spectra.

Results

Hydrophobicity plot of LtnT homologues. LtnT, the transporter of lacticin 3147, belongs to the family of ABC transporter maturation and secretion (AMS) proteins. It is 708 amino acids long, containing a N-terminal peptidase domain, a central transmembrane domain and a C-terminal cytosolic ATP-binding domain (figure 1A). Studies of Franke et al. (3) and Nishie et al. (4) indicate that the peptidase domain also localizes at the cytoplasmic side of the membrane. To facilitate a cytoplasmic location for both ATPase domain as well as the peptidase domain, there must be an even number of transmembrane helices. However it is not yet clear if the central transmembrane domain contains four or six helices. Therefore, 120 homologues of LtnT (supplementary table 1) where used to generate an averaged hydrophobicity plot using MemGen 4.2.0 (14). The averaged hydrophobicity plot (figure 1B) clearly shows the presence of six transmembrane helices and confirms the previously proposed localization of the N- and C-terminal domains.

Multiple sequence alignment of AMS proteins. To assess the role of the peptidase domain in transport, a multiple sequence alignment was generated to identify conserved residues of the active site. LtnT was aligned against known AMS proteins, SunT, NukT, LctT, CvaB and ComA (figure 2). This revealed a number of conserved residues, i.e., Gln9, which is part of the assumed oxyanion hole, and Cys15, His89 and Asp105 that localize to the predicted catalytic site. Based on these alignment results, a variant of LtnT was created by site-directed mutagenesis in which the conserved cysteine at position 15 was exchanged for an alanine. For a second variant of LtnT, the peptidase C39 encoding sequence was removed. BLAST analysis suggests that this domain of LtnT is specified by

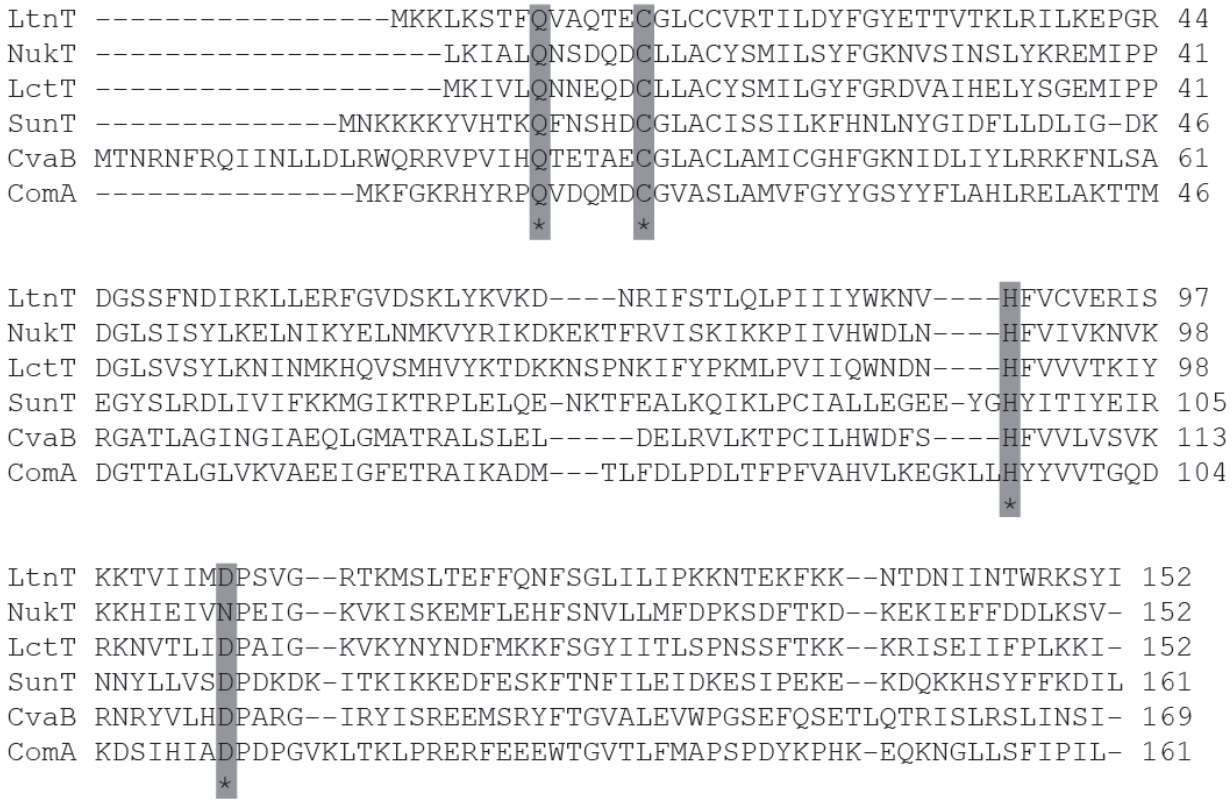

Figure 2: Multi sequence alignment of the first 152 amino acids of LtnT (NCBI accession number: AAC56012.1) against NukT (BAC98761.2), LctT (AAC72259.1), SunT (AAC12993.1), CvaB (CAA40744.1) and ComA (AAA69510.1). The conserved residues of the peptidase C39 domain are marked with *.

amino acids 4 till 129. To ensure the complete removal, residues Lys3 till Glu134 were deleted.

Expression of LtnT and variants in *L. lactis* NZ9000. In order to determine if the LtnT transporter variants are expressed in *L. lactis* NZ9000, a His-tag sequence was introduced at the C-terminus of the ATP binding domain. After expression, cell samples were compared to an empty vector control by SDS-PAGE. His-LtnT and His-LtnT-C15A could be detected as an unique protein around 75 kDa that was absent when cells were transformed with the control plasmid (figure 3A). The truncate His-trLtnT could not be assigned in coomassie brilliant blue stained SDS-PAGE. Therefore, samples were also analyzed by Western blotting using a His-tag antibody. This allowed for the detection of His-tagged proteins of about 75 kDa, corresponding to His-LtnT and His-LtnT-C15A, and of 60 kDa, which corresponds to the His-trLtnT (figure 3B). The proteins

show a faster migration on SDS-PAGE than expected for their calculated masses of 80.8 and 65.5 kDa, respectively. This is a typical feature of membrane proteins. In addition, some C-terminal degradation products of LtnT were observed that were absent in the empty vector control. The data demonstrate that LtnT and respective variants are expressed in *L. lactis* NZ9000.

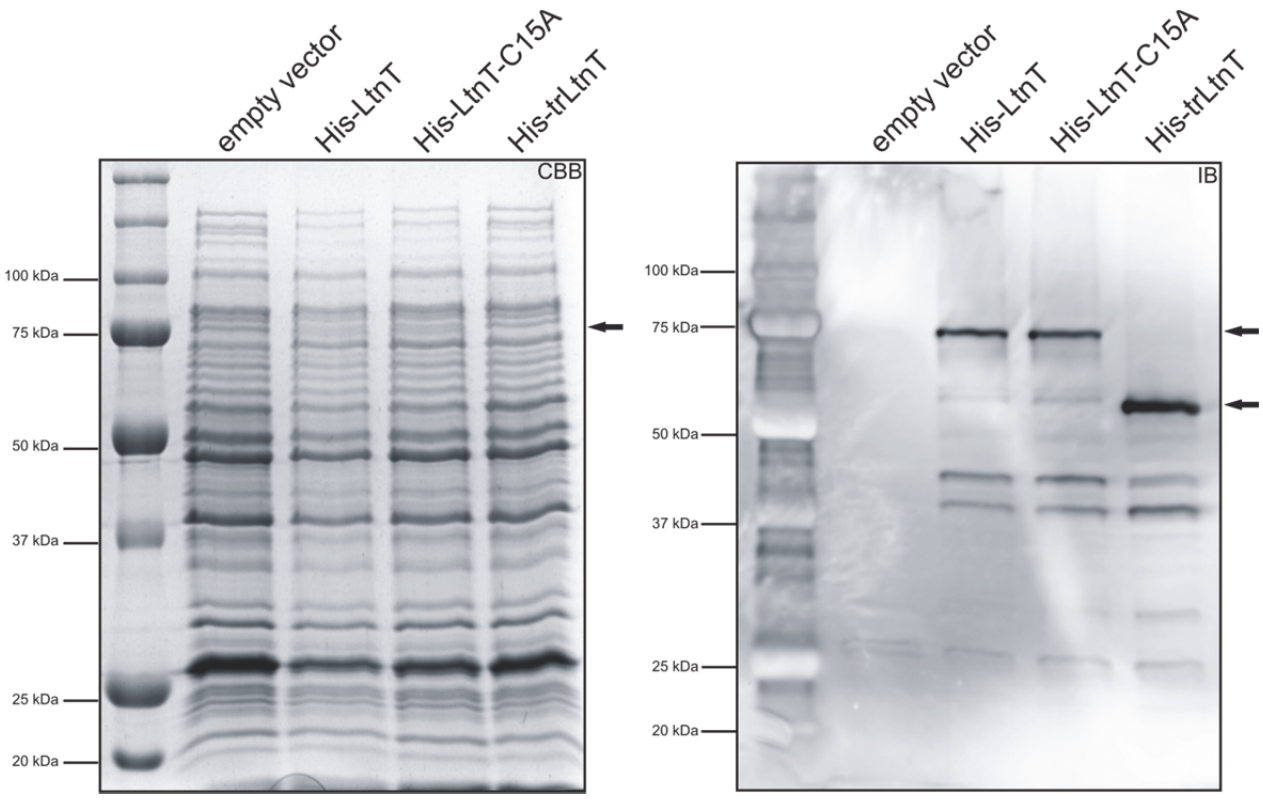

Figure 3: A) Coomassie stained SDS-PAGE comparing cells of *L. lactis* strains expressing His-LtnT, His-LtnT-C15A, His-trLtnT or the empty vector. The arrow indicates an unique protein at 75 kDa. B) Western blot stained with a Tetra-His antibody (Qiagen) showing by an arrow the presence of His-tagged LtnT and LtnT-C15A at 75 kDa and trLtnT at 60 kDa.

Secretion and processing of LtnA1 and LtnA2 by LtnT and variants. In order to evaluate if LtnT is active without the presence of the associated biosynthesis enzymes of the lacticin 3147 gene cluster, LtnT was co-expressed with either LtnA1 or LtnA2. The supernatant of the expressing strains was screened via MALDI-TOF mass spectrometry for the presence of processed but unmodified LtnA1 and LtnA2. A peak of 3424.59 Da (figure 4A) was observed in the supernatant of the strain co-expressing LtnT and LtnA1 that was absent in the empty vector controls. The observed mass corresponds to the theoretical

mass of 3430.88 Da (15) for the unmodified core peptide of LtnA1. Likewise, co-expression of LtnA2 and LtnT yielded a peptide with a mass of 2982.85 Da (figure 4B). This mass corresponds to the theoretical mass of unmodified processed LtnA2, 2986.44 Da (15). Taken together the results show that the lacticin 3147 transporter can transport and process its unmodified natural substrates, albeit at low levels.

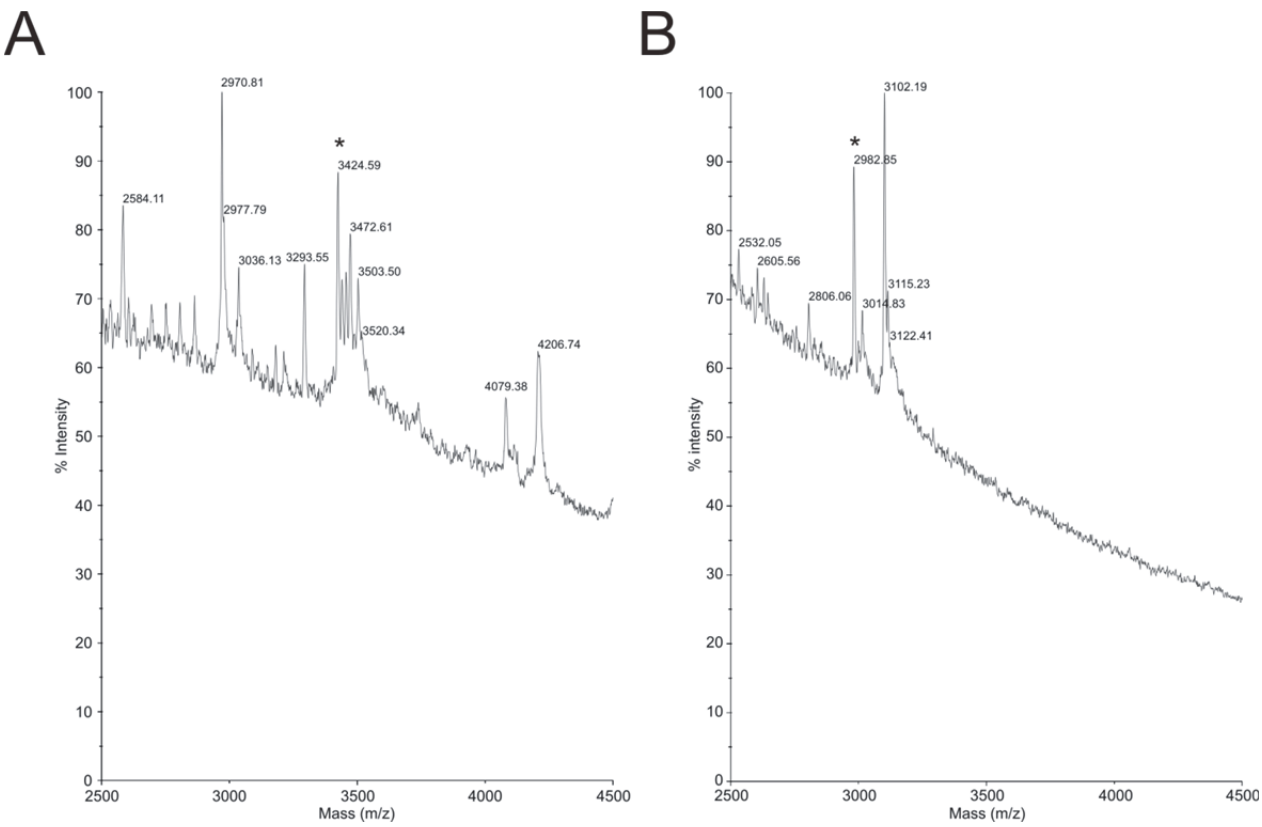

Figure 4: MALDI-TOF mass spectra of culture media showing the presence of (A) unmodified core peptide LtnA1 of 3424.59 Da after co-expression of LtnA1 and LtnT in *L. lactis* NZ9000, (B) unmodified core peptide LtnA2 at 2982.85 Da after co-expression of LtnA2 and LtnT in *L. lactis* NZ9000.

Next, the ability of the LtnT transporter containing the cysteine to alanine mutation at position 15, to transport LtnA1 or LtnA2 was analyzed. However, in this case, no peptide product could be detected. Also with the second variant of LtnT with a truncation of the peptidase C39 domain no peptide product of LtnA1 or LtnA2 could be detected in the medium fraction. These results suggest that

processing of the lanthipeptides is an important requirement for LtnT mediated export.

Discussion

Since LtnT is responsible for both the maturation and the secretion of LtnA1 and LtnA2, it plays a key role in the biosynthesis of lacticin 3147. Previous reports on NukT (16) showed that NukT is not able to transport unmodified peptides. Here it is shown that co-expression of LtnT with either LtnA1 or LtnA2 does results in the production of unmodified and processed peptides. This suggests that LtnT can function without the presence of other enzymes encoded in the gene cluster of lacticin 3147, and thus that modification is not an essential requirement for LtnT mediated transport and processing. However, it is noted that the level of production is low causing a high background in the MALDI-TOF mass spectra, thus indicating impaired transport.

A BLAST analysis revealed that LtnT contains a conserved peptidase domain that belongs to the peptidase C39 family. By aligning this domain with peptidase domains of other AMS proteins, conserved amino acids were identified in LtnT (figure 2). Although various studies (3-9, 16) report work on AMS proteins and their peptidase domain, the exact role of the peptidase C39 domain in the transport mechanism of the lacticin 3147 transporter is unknown. Therefore, two variants of LtnT, LtnT-C15A with an alanine replacement of the conserved catalytic cysteine residue, and a truncate of the peptidase domain, trLtnT, were assessed for their ability to export LtnA1 and LtnA2. The truncate more resembles transporters associated with class I lantibiotics that secrete the peptides independently from their processing. However, eliminating the active cleavage site either by mutation or deletion completely abolished the production and likely the export. This suggests that LtnT requires an active peptidase C39 domain in order to export peptides. Similar findings were reported for the lantibiotic transporter NukT (4), and it was suggested that the transport and processing are cooperative events.

Taken together, this study shows that unmodified substrate peptides can be processed and exported by LtnT. In addition, LtnT requires an active peptidase C39 domain to transport substrates across the membrane.

Table 3: Peptide sequence of the expressed peptides and their theoretical mass.

	Lantibiotic leader peptide	Lantibiotic core peptide	Mass (Da) precursor peptide*	Mass (Da) core peptide**
	-25 -20 -15 -10 -5 -1	1 5 10 15 20 25 30		
LtnA1	MNKNEIETQPVIWLEEVSDQNFDEDVFGA	CSTNTFSLSDYWGNNGAWCTLTHECMAWCK	6668.24	3430.88 (15)
	-35 -30 -25 -20 -15 -10 -5 -1	1 5 10 15 20 25		
LtnA2	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG	TTPATPAISILSAYISTNTCPTTKCTRAC	6949.84	2986.44 (15)

* The average mass of the proteins, $(M+H)^+$, was calculated without the initial methionine.
** The monoisotopic mass of the peptides $(M+H)^+$

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Supplementary information

Supplementary table 1: List of the 120 proteins used to create the hydrophobicity plot for the ABC transporter maturation and secretion (AMS) protein family including LtnT.

Protein name in MemGen 4.2.0	GI number	NCBI-definition
LCGT1755lgar	347522361	ABC transporter ATP binding protein/permease [*Lactococcus garvieae* ATCC 49156]
MCHL5208mext	218533082	ABC transporter [*Methylobacterium extorquens* CM4]
HEL16410shel	257064337	multidrug ABC transporter ATPase and permease [*Slackia heliotrinireducens* DSM 20476]
BVU0291bavu	150002889	ABC transporter ATP-binding protein [*Bacteroides vulgatus* ATCC 8482]
MTOL0119eoli	408671693	ABC transporter related protein [*Emticicia oligotrophica* DSM 17448]
FP1877fpsy	150025919	Probable ABC-type multidrug transport system, ATPase and permease components [*Flavobacterium psychrophilum* JIP02/86]
63030342cals	428297090	bacteriocin-processing peptidase [*Calothrix* sp. PCC 6303]
AVA2398avar	75908614	bacteriocin-processing peptidase [*Anabaena variabilis* ATCC 29413]
FJOH1164fjoh	146298925	ABC transporter-like protein [*Flavobacterium johnsoniae* UW101]
ACSA3723bsal	324977005	ABC-type bacteriocin transporter [*Bacteroides salanitronis* DSM 18170]
PH214095sspc	326801479	xenobiotic ABC transporter ATPase [*Sphingobacterium* sp. 21]
ALHY2553hahy	332664511	xenobiotic-transporting ATPase [*Haliscomenobacter hydrossis* DSM 1100]
DFER4910dfer	255038655	ABC transporter [*Dyadobacter fermentans* DSM 18053]
LAGDfaes	436836888	Lactococcin-G-processing and transport ATP-binding protein lagD [*Fibrella aestuarina* BUZ 2]
BREC0941erec	238923324	transporter, truncation, partial [*Eubacterium rectale* ATCC 33656]
OCEL0740ccel	302873642	bacteriocin ABC transporter [*Clostridium cellulovorans* 743B]
REBR1063tbre	332297704	bacteriocin ABC transporter [*Treponema brennaborense* DSM 12168]
TDE0425tden	42525941	bacteriocin ABC transporter ATP-binding/permease [*Treponema denticola* ATCC 35405]
SPSE0465stps	386318478	ABC-type bacteriocin transporter family protein [*Staphylococcus pseudintermedius* ED99]
BALH4659bthu	118480203	ABC-type bacteriocin transporter family protein [*Bacillus thuringiensis* str. Al Hakam]
CTHE0534rthe	125973053	bacteriocin-processing peptidase [*Ruminiclostridium thermocellum* ATCC 27405]
CAP0073cace	15004777	ABC ATPase containing transporter [*Clostridium acetobutylicum* ATCC 824]
ALHA1929halh	435854614	ABC-type bacteriocin transporter [*Halobacteroides halobius* DSM 5150]
CSAC0158csac	146295230	ABC-type bacteriocin transporter [*Caldicellulosiruptor saccharolyticus* DSM 8903]
AMET1032alme	150388859	bacteriocin ABC transporter [*Alkaliphilus metalliredigens* QYMF]
62PC0024efae	384519720	bacteriocin ABC transporter [*Enterococcus faecalis* 62]
P6700602spne	307126742	transport/processing ATP-binding protein ComA [*Streptococcus pneumoniae* 670-6B]
LJP0571ljoh	385825511	lactacin F ABC transporter permease component [*Lactobacillus johnsonii* DPC 6026]
SUNTlegl	289164872	ABC-type bacteriocin/lantibiotic exporters, contain an N-terminal double-glycine peptidase domain [*Legionella longbeachae* NSW150]
BPRI0803bupr	302670168	bacteriocin ABC transporter ATP-binding protein/permease/protease [*Butyrivibrio proteoclasticus* B316]
SWOL2230swol	114567738	hypothetical protein Swol_2230 [*Syntrophomonas wolfei* subsp. *wolfei* str. Goettingen G311]
ELEN2946elen	257792673	ABC transporter-like protein [*Eggerthella lenta* DSM 2243]
YAGR0273cygr	427701593	NHLM bacteriocin system ABC transporter, peptidase/ATP-binding protein [*Cyanobium gracile* PCC 6307]

Protein name in MemGen 4.2.0	GI number	NCBI-definition
28316118mrad	170745389	ABC transporter [*Methylobacterium radiotolerans* JCM 2831]
PUNR1803npun	186682212	ABC transporter [*Nostoc punctiforme* PCC 73102]
PUNF5050npun	186685135	ABC transporter [*Nostoc punctiforme* PCC 73102]
NACY1965acyl	440681565	NHPM bacteriocin system ABC transporter, peptidase/ATP-binding protein [*Anabaena cylindrica* PCC 7122]
GMSR0644mgry	568145045	ABC transporter related protein [*Magnetospirillum gryphiswaldense* MSR-1]
ALHY0667hahy	332662660	NHPM bacteriocin system ABC transporter, peptidase/ATP-binding protein [*Haliscomenobacter hydrossis* DSM 1100]
SUNTpthe	147678715	bacteriocin/lantibiotic ABC transporter [*Pelotomaculum thermopropionicum* SI]
PLUT0873clut	78186738	ATPase [*Chlorobium luteolum* DSM 273]
XNC14505xnem	300725260	Colicin V secretion/processing ATP-binding protein cvaB [*Xenorhabdus nematophila* ATCC 19061]
A386B254apas	529230277	ABC transporter related [*Acetobacter pasteurianus* 386B]
ARI03623saen	161505465	hypothetical protein SARI_03623 [*Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23:- str. RSK2980]
S7000270prst	386741469	hypothetical protein S70_00270 [*Providencia stuartii* MRSN 2154]
17580105ypse	153949267	bacteriocin ABC transporter ATP-binding-protein/permease [*Yersinia pseudotuberculosis* IP 31758]
KVAR4715kvar	288937560	ABC transporter [*Klebsiella variicola* At-22]
SWP1032spie	212633897	Toxin secretion ATP-binding protein [*Shewanella piezotolerans* WP3]
CJA3604ceja	192361880	putative toxin transporter [*Cellvibrio japonicus* Ueda107]
AS122274sers	333927129	peptide-transporting ATPase [*Serratia* sp. AS12]
CC0684ccre	16124937	ABC transporter [*Caulobacter crescentus* CB15]
ARAD7449arad	222081179	toxin secretion ABC transporter [*Agrobacterium radiobacter* K84]
RLEG6343rleg	241554125	ABC transporter [*Rhizobium leguminosarum* bv. *trifolii* WSM1325]
CVABpvag	308185474	type I secretion system atpase [*Pantoea vagans* C9-1]
MCHFsply	518650451	putative microcin-H47 secretion/processing ATP-binding protein MchF [*Serratia plymuthica* 4Rx13]
SDEN0474sden	91791838	ABC transporter related [*Shewanella denitrificans* OS217]
RAXB1xalb	285018564	peptide ABC transporter ATPase/permease [*Xanthomonas albilineans* GPE PC73]
SWOO3807swoo	170728135	ABC transporter-like protein [*Shewanella woodyi* ATCC 51908]
SESU1940psuw	319787533	ABC transporter [*Pseudoxanthomonas suwonensis* 11-1]
BPRA0247ppro	54307468	toxin transporter [*Photobacterium profundum* SS9]
SOA0049sone	24376277	toxin secretion ABC transporter, ATP-binding subunit/permease protein, putative [*Shewanella oneidensis* MR-1]
SWP4866spie	212637550	Toxin secretion ABC transporter, ATP-binding subunit/permease protein, putative [*Shewanella piezotolerans* WP3]
BNIB1807vnig	549719946	putative COLICIN V SECRETION ABC TRANSPORTER [*Vibrio nigripulchritudo*]
PECL1942pecl	381280280	ABC-type bacteriocin transporter [*Pediococcus claussenii* ATCC BAA-344]
ELEN2945elen	257792672	ABC transporter-like protein [*Eggerthella lenta* DSM 2243]
ENT29170ents	479181661	ABC-type bacteriocin/lantibiotic exporters, contain an N-terminal double-glycine peptidase domain [*Enterococcus* sp. 7L76]
73311066rode	311112735	cytolysin B transport protein [*Rothia dentocariosa* ATCC 17931]
LKTBrode	311112748	peptide-transporting ATPase [*Rothia dentocariosa* ATCC 17931]
GS508715smut	397650480	Toxin RTX-I translocation ATP-binding protein [*Streptococcus mutans* GS-5]
GTNG2061gthe	138895705	lantibiotic ABC transporter [*Geobacillus thermodenitrificans* NG80-2]
BH0451bhal	15613014	lantibiotic mersacidin transporter system [*Bacillus halodurans* C-125]
LANTblic	511061028	lantibiotic transport protein LanT [*Bacillus licheniformis* 9945A]
ATHE1112cbes	222529104	ABC transporter [*Caldicellulosiruptor bescii* DSM 6725]
APXIBcsai	550920830	toxin RTX-I translocation ATP-binding protein [*Clostridium saccharobutylicum* DSM 13864]
OCEL4224ccel	302877016	peptidase C39 bacteriocin processing [*Clostridium cellulovorans* 743B]
OCEL4227ccel	302877019	peptidase C39 bacteriocin processing [*Clostridium cellulovorans* 743B]

Protein name in MemGen 4.2.0	GI number	NCBI-definition
GAU3887gaur	226229293	putative bacteriocin processing/transport ATP-binding protein [*Gemmatimonas aurantiaca* T-27]
L0835646acti	494688062	ABC transporter [*Actinoplanes* sp. N902-109]
HAUR1869haur	159898393	ABC transporter-like protein [*Herpetosiphon aurantiacus* DSM 785]
HAUR3741haur	159900258	ABC transporter-like protein [*Herpetosiphon aurantiacus* DSM 785]
66052216cmin	434386215	ABC-type bacteriocin/lantibiotic exporter with N-terminal double-glycine peptidase domain [*Chamaesiphon minutus* PCC 6605]
E1093122anas	153005977	ABC transporter-like protein [*Anaeromyxobacter* sp. Fw109-5]
PAXBcoco	383455645	exotoxin translocation ATP-binding protein PaxB [*Corallococcus coralloides* DSM 2259]
HOCH5839hoch	262198999	ABC transporter [*Haliangium ochraceum* DSM 14365]
MCHFcoco	383457198	putative lantibiotic ABC transporter permease/ATP-binding protein [*Corallococcus coralloides* DSM 2259]
MXAN2853mxan	108761029	lantibiotic ABC transporter permease/ATP-binding protein [*Myxococcus xanthus* DK 1622]
TAUR3272stia	310820533	antibiotic ABC transporter permease/ATP-binding protein [*Stigmatella aurantiaca* DW4/3-1]
20002102pmir	529237769	toxin transporter [*Proteus mirabilis* BB2000]
VV20484vvul	27366918	RTX toxin transporter [*Vibrio vulnificus* CMCP6]
PLU3127plum	37527015	hypothetical protein plu3127 [*Photorhabdus luminescens* subsp. *laumondii* TTO1]
YE1998yent	123442269	RTX family toxin transporter [*Yersinia enterocolitica* subsp. *enterocolitica* 8081]
RTXBpasy	253990551	RTX toxin ABC transporter protein [*Photorhabdus asymbiotica*]
OCEL2848ccel	302875675	type I secretion system ATPase [*Clostridium cellulovorans* 743B]
SSA1100ssan	125717929	hemolysin exporter, ATPase component [*Streptococcus sanguinis* SK36]
38901580falo	374308022	leukotoxin translocation ATP-binding protein LktB [*Filifactor alocis* ATCC 35896]
B3811446caho	154147938	leukotoxin translocation ATP-binding protein LktB [*Campylobacter hominis* ATCC BAA-381]
ULKU1197skuj	313682322	type I secretion system ATPase [*Sulfuricurvum kujiense* DSM 16994]
01EI2150eict	238920046	type I secretion system ATPase family protein [*Edwardsiella ictaluri* 93-146]
SMED0884smed	150396107	type I secretion system ATPase [*Sinorhizobium medicae* WSM419]
M4463820mspm	170741976	type I secretion system ATPase [*Methylobacterium* sp. 4-46]
AFE2693acfe	218665057	type I secretion system ATPase [*Acidithiobacillus ferrooxidans* ATCC 23270]
D7812189smar	440230852	type I secretion system ABC transporter, HlyB family [*Serratia marcescens* FGI94]
30165030pmuc	379722776	hypothetical protein PM3016_5030 [*Paenibacillus mucilaginosus* 3016]
PAJ1258pana	386015850	hypothetical protein PAJ_1258 [*Pantoea ananatis* AJ13355]
NMCC1317nmen	161870276	ABC transporter family protein [*Neisseria meningitidis* 053442]
PPRO2369pepr	118580783	type I secretion system ATPase [*Pelobacter propionicus* DSM 2379]
VEIS2576veis	121609532	type I secretion system ATPase [*Verminephrobacter eiseniae* EF01-2]
35231035fcf.	387824618	ABC-type bacteriocin/lantibiotic exporter [*Francisella cf. tularensis* subsp. *novicida* 3523]
BPRO1020posp	91786919	Type I secretion system ATPase, HlyB [*Polaromonas* sp. JS666]
SVI3379svio	294142150	toxin secretion ABC transporter protein, HlyB family [*Shewanella violacea* DSS12]
SY112340ffas	479198404	type I secretion system ABC transporter, HlyB family [*Fretibacterium fastidiosum*]
HLYBspja	294013160	hemolysin secretion protein HlyB [*Sphingobium japonicum* UT26S]
ESAL2073dsal	242279543	type I secretion system ATPase [*Desulfovibrio salexigens* DSM 2638]
CAUL4165caus	167648124	type I secretion system ATPase [*Caulobacter* sp. K31]
ESOP5579mopp	337270029	type I secretion system ATPase [*Mesorhizobium opportunistum* WSM2075]
MMC11970magn	117925265	type I secretion system ATPase [*Magnetococcus marinus* MC-1]

Protein name in MemGen 4.2.0	GI number	NCBI-definition
RADO6926brsp	146343683	toxin secretion ABC transporter ATP-binding and membrane protein [*Bradyrhizobium* sp. ORS 278]
HLYBndef	302038746	type I secretion system AtPase HlyB [*Candidatus Nitrospira defluvii*]
RPE1085rpal	115523107	type I secretion system ATPase [*Rhodopseudomonas palustris* BisA53]
RADO6201brsp	146343016	toxin secretion ABC transporter ATP-binding and membrane protein [*Bradyrhizobium* sp. ORS 278]

Chapter 3

Production of the class II lantibiotic lacticin 3147 in *Lactococcus lactis* NZ9000

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Abstract

Lantibiotics are ribosomal synthesized peptides that contain unusual amino acids and that are stabilized by thioether bonds. In class II lantibiotics, these post-translational modifications involve a dehydratase and cyclase activity both catalyzed by a single bifunctional enzyme, LanM. Production of the two component lantibiotic lacticin 3147 depends on the activity of LtnM1 and LtnM2 lanthionine synthetase enzymes and the transporter LtnT. To examine the performance of these enzymes as a generic class II lantibiotic production system, the respective genes were co-expressed in *Lactococcus lactis* NZ9000. LtnM1 and LtnM2 in conjunction with LtnT supported the modification, maturation and excretion of the cognate substrates LtnA1 and LtnA2, respectively. When mixed, LtnA1 and LtnA2 inhibited the growth of an indicator strain demonstrating the production of the active two-component lantibiotic.

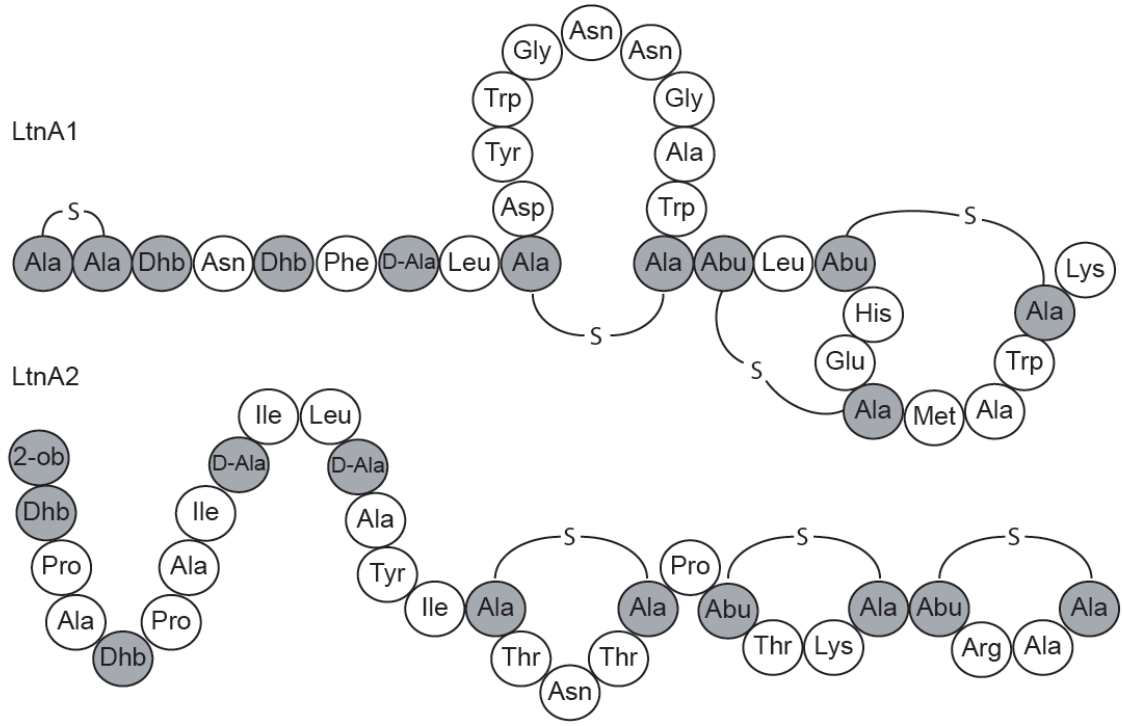

Figure 1: shorthand notation of mature LtnA1 and LtnA2 of the two-component lantibiotic lacticin 3147 (after the example from Cotter et al. (22)). The modified residues are marked in gray.

Introduction

The two-component lantibiotic lacticin 3147 (figure 1) is produced by *Lactococcus lactis* subsp. *lactis* DPC3147 (1). Lantibiotics are a subgroup of lanthipeptides that display antimicrobial activity. Lanthipeptides are ribosomal synthesized and post-translationally modified peptides that contain the unusual amino acid *meso*-lantihionine (Lan) and (*2S,3S,6R*)-3-methyllanthionine (MeLan). These amino acids are formed during the post-translational modification of serine and threonine residues that are dehydrated to form 2,3-dehydroalanine or (*Z*)-2,3-dehydrobutyrine, respectively. Dehydroalanine or dehydrobutyrine residues are then coupled to cysteine via a thioether bond to form the lanthionine or methyllanthionine ring, respectively. Based on the modification enzymes that introduces the (methyl)lanthionine residues, lanthipeptides can be divided into four classes (2). Class I and II lanthipeptides are mainly comprised of the more well-known lantibiotics, lanthionine-containing antibiotic peptides. In class I lanthipeptides, or lantibiotics, the formation of a

lanthionine ring is catalyzed by separate dehydratase (LanB) and cyclase (LanC) enzymes, whereas in class II lantibiotics both reactions are catalyzed by a single bifunctional enzyme, LanM. LanM enzymes typically contain a N-terminal dehydratase domain which does not show any homology to LanB. The C-terminal domain of LanM contains a cyclase domain which shows ~25% sequence identity to LanC (2), including the conserved zinc-binding residues that are required for catalysis (3). Class III and IV lanthipeptides also contain a single multifunctional modification enzymes to install the (methyl)lanthionine rings, LanKC and LanL, respectively (4, 5). Both enzymes contain three domains, a N-terminal lyase domain, a central kinase domain and a C-terminal cyclase domain. The difference between the LanKC and LanL enzyme lies within the cyclase domain. The cyclase domain of LanL enzymes is homologous to LanC while the cyclase domain of LanKC appears unique. In addition, some LanKC enzymes are also able to form a so-called labionin structure in which a second dehydroalanine is added to the enolate intermediate, formed by the addition of a cysteine to a dehydroalanine residue (6).

The genes involved in lantibiotic biosynthesis are usually found in clusters. These clusters encode genes for the modification enzymes (LanBC or LanM). In addition to the (methyl)lantihonine residue forming enzymes, lantibiotics can undergo further post-translational modifications like decarboxylation (LanD) or in the case of lacticin 3147 conversion of L-serine to D-alanine catalyzed by LtnJ. Furthermore, the clusters also contain genes encoding an ATP-binding cassette (ABC) transporter (*lanT*) to transport the peptide into the environment. In class II lantibiotics these transporters contain a proteolytic domain to remove the leader peptide, however class I lantibiotic transporters do not contain such a domain instead encode a protease that cleaves the leader sequence (*lanP*) from the secreted precursor peptide. The gene cluster furthermore contains genes encoding proteins involved in self-protection/immunity (*lanIFEG*) and regulation of lantibiotic production (*lanKR*). The *lanA* gene encodes the linear precursor peptide. Lacticin 3147 is a two-component lantibiotic and its functional lanthipeptide is formed by two peptides encoded in the gene cluster as two precursor peptides, *ltnA1* and *ltnA2*. Both precursor peptides depend on a dedicated modification enzyme, LtnM1 and LtnM2, respectively (7). The lantibiotic precursor peptide contains at its N-terminus a leader peptide that is recognized by the modification enzymes and transporter. The C-terminal core

peptide is modified and after leader peptide removal, it is released into the medium as a mature lantibiotic. In class II lantibiotics, the leader sequence contains a double glycine motif, GG, GA, GS or GT, which is recognized by the N-terminal peptidase C39 domain of the bifunctional transporter typically found in class II lantibiotics.

Antibiotic resistance is currently a serious threat in the treatment of infectious diseases. Therefore, novel antibiotics based on new core structures are urgently needed to counteract this resistance. Lantibiotics are an interesting group of molecules with the potential to act as antimicrobial agents. So far no known resistance mechanism exists against lantibiotics other than the immunity proteins found in the producing strains. For instance, the lantibiotic nisin is used for over 50 years as a food preservative without the significant development of microbial resistance. The antimicrobial activity of a few lantibiotics has been studied in detail, and these peptides act by inhibiting cell wall biosynthesis by binding lipid II, disrupting membrane integrity by forming pores, or a combination of the two mechanisms (2). With the two-component lacticin 3147, both peptides need to be present at an equimolar concentration (9, 10) to yield the active unit. LtnA1 forms a complex with lipid II thereby inhibiting cell wall biosynthesis. LtnA2 will then bind this complex and the complex will then form pores in the membrane (11).

A large arsenal of lanthipeptides producing gene clusters has been found in microbial genomes and these reflect a so far untapped source of bioactivity. To produce these lanthipeptides in the natural host is not a simple task as often the conditions for production and growth are not known, nor has the host been isolated or cultivated. The development of a heterologous expression system to produce so far uncharacterized lanthipeptides might provide a generic solution to this problem. Such a system has been developed for class I lanthipeptides based on the nisin gene cluster employing the modification enzymes (NisBC) and the transporter (NisT) of nisin (12-16). However, for class II lanthipeptides, a generic production system does not yet exist. In a previous study it was shown that LtnM2 and LtnT can modify, process and transport LtnA2 and unrelated peptides, containing nisin and angiotensin variants fused behind the leader peptide of lacticin A2 (17). Interestingly, for LtnM1 and LtnT it is unclear if they are able to modify, process and transport LtnA1 separate from remaining

enzymes encoded in the lacticin 3147 gene cluster. Therefore, the potential of LtnM1 and LtnT to produce functionally active LtnA1 was examined in this study.

Materials and methods

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in table 1. *Lactococcus lactis* NZ9000 was used as a host for the plasmids. All strains were grown at 30°C in Bacto M17 broth (Becton Dickinson Difco) supplemented with 0.5% glucose. Where needed chloramphenicol (5 µg/mL), erythromycin (5 µg/mL) or both antibiotics (3 µg/mL) were added to the medium.

To create the expression strains the plasmids pA13 or pA24 were transformed to *L. lactis* NZ9000 in combination with pILPM1T or pILPTM2, respectively, by means of electrotransformation as described previously by Holo et al. (18) using a Bio-Rad gene pulser.

Table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
strains		
Lactococcus lactis NZ9000	MG1363 derivative; pepN::nisRK+	(19)
L. lactis LL108 (pORI 208)	Em^r Cm^r	(19, 20)
L. lactis IFPL105	pBAC105, lacticin 3147 producing strain	(25)
L. lactis subsp. *lactis* IL1403		
Plasmids		
pIL253	Em^r	(26)
pILPTM2	pIL253 derived; *ltnT-ltnM2* cloned behind the *Pnis* promoter; Em^r	(17) Kind gift from Lanthio Pharma
pILPM1T	pIL253 derived; *ltnM1-ltnT* cloned behind the *Pnis* promoter; Em^r	Kind gift from Lanthio Pharma
pNZ8048	Cm^r	(19)
pA24	pNZ8048 derived; *ltnA2* cloned behind the *Pnis* promoter; Cm^r	(17) Kind gift from Lanthio Pharma
pA13	pNZ8048 derived; *ltnA1* cloned behind the *Pnis* promoter; Cm^r	Kind gift from Lanthio Pharma

Em^r erythromycin resistance marker
Cm^r chloramphenicol resistance marker

SDS-PAGE analysis of LtnM1 and LtnM2. *L. lactis* NZ9000 cells were transformed with pILPM1T & pA13 or pILPTM2 & pA24 and grown overnight in GM17. Fresh GM17 was inoculated 1:60 with an overnight culture of the expression strain, incubated for 3 hours at 30°C (optical density at 600 nm (OD_{600}) between 0.4-0.5) till expression was induced by adding nisin (Sigma-Aldrich) with an end concentration of 1 ng/mL. After 4 hours of induction cells were harvested by centrifugation (16,100 x g for 8 minutes) and cell pellets were kept at -20°C. Before the sample was applied to SDS-PAGE the cell pellet was lysed by resuspension in 4 mg/mL Lysozyme (Roche) in MilliQ water and incubation for 25 min. at 55°C. By means of SDS-PAGE and Coomassie brilliant blue staining expression of the lacticin 3147 modification enzymes was assessed.

MALDI-TOF mass spectrometry. *L. lactis* expression strains were grown overnight in GM17 and diluted 50-fold in minimal medium (14). Cultures were grown for 3 hours at 30°C before expression was induced by adding nisin (Sigma-Aldrich) to an end concentration of 1 ng/mL. After 4 (and 24) hours of incubation, the supernatant was collected by centrifugation (20 min. at 3724 x g). Peptides were precipitated from the supernatant by the addition of 10% trichloroacetic acid (TCA) and incubation on ice for 30 min. Next, the sample was spun for 30 min. at 17,000 x g and the pellet was washed with acetone. To remove the acetone, the sample was spun 15 min. at 17,000 x g and dried for 5 min. at 42°C. TCA precipitated material was dissolved in 10 µL MilliQ water, and 1 µL of the sample was applied to the MALDI-TOF target, dried and washed with a drop of MilliQ water. Subsequently, 1 µL of matrix, 5 mg/mL α-Cyano-4-hydroxycinnamic acid (Sigma-Aldrich) dissolved in 50% acetonitrile and 0,1% trifluoroacetic acid (TFA), was added to the sample and dried. MALDI-TOF mass spectrometry spectra were recorded with a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems) and analyzed with Data Explorer TM 4.0.0.0 (Applied Biosystems).

Antimicrobial activity assays. *L. lactis* expression strains overnight grown in GM17 were diluted 60-fold in fresh GM17 medium and incubated at 30°C until it reached an optical density at 600 nm (OD_{600}) between 0.4-0.6. The expression was induced by adding nisin (Sigma-Aldrich) to an end concentration of 1 ng/mL. After 4 hours of incubation the cells were removed from the

supernatant by centrifugation (20 min. at 3724 x g). The sample was used for TCA precipitation as described above. The TCA precipitated material was dissolved in GM17, with appropriate antibiotics, to create a 1-time or 4-times concentrated sample.

For the activity assay the 1-time concentrated TCA precipitated samples were used to create a two-fold diluted series in a microtiter-plate. These samples were overlaid in an one to one ratio with an indicator strain. After 5 hours of incubation at 30°C the growth inhibition was measured by measuring the OD_{600}. The indicator strains, *L. lactis* LL108(pORI 280) (19, 20) and *L. lactis* subsp. *lactis* IL1403, were prepared as followed; an overnight culture of the indicator strain was diluted 100-fold in fresh medium and grown at 30°C until OD_{600} reached 0.2-0.3 before added to the lanthipeptide samples.

In addition, the bioactivity was assessed by the use of a well-diffusion assay. An overnight culture of the indicator strains was diluted 800-times in warm (45°C) 0.7% GM17-agar (with appropriate antibiotics). This was used to pour a top layer of 16 mL onto plates containing a layer of 10 mL 1.5% GM17-agar (with appropriate antibiotics). Wells of 8 mm were formed and a volume of 50 µL 4-times concentrated TCA precipitated lanthipeptides was dispensed in the wells. After one hour incubation at room temperature the plates were placed overnight at 30°C, and growth inhibition was determined by the presence of an inhibition zone.

Results

Co-expression of LtnM1, LtnT and LtnA1 in *Lactococcus lactis*. The lantibiotic lacticin 3147 is a so called two-component lantibiotic. It is composed out of two structural entities LtnA1 and LtnA2. Both components are modified by a dedicated modification enzyme, LtnM1 and LtnM2, respectively, but are processed and exported by a single transporter, LtnT. Previously, it was shown that the transporter could be expressed in *L. lactis* NZ9000 and retained its export and process function while expressed without the remaining lacticin 3147 enzymes (Chapter 2). To assess the ability of the enzymes LtnM1 and LtnT to modify, process and transport class II lanthipeptides as a generic production system, the respective genes were co-expressed with the LtnA1 encoding gene, as substrate under control of the nisin promoter, in *L. lactis* NZ9000. First the

expression of LtnM1 in *L. lactis* NZ9000 by means of SDS-PAGE was assessed. cells co-expressing LtnM1, LtnT and its substrate were examined on a Coomassie brilliant blue stained SDS-PAGE. The theoretical mass of LtnM1 is 111,749 Da, however no protein band suggesting the presence of LtnM1 was seen on SDS-PAGE (figure 2). When supernatant of the culture was collected, subjected to TCA precipitation and analyzed by MALDI-TOF mass spectrometry a product of 3301.62 Da was yielded (figure 3A) not found with the control strain transformed with an empty expression plasmid (data not shown). The unique mass corresponds to the theoretical mass of LtnA1 that is seven times dehydrated, i.e., 3304.74 Da (21). The absence of detectable LtnM1 on SDS-PAGE indicates a low expression level but the MALDI-TOF mass spectrometry data suggest that dehydrated and processed LtnA1 was produced when LtnA1, LtnM1 and LtnT are co-expressed in *L. lactis* NZ9000.

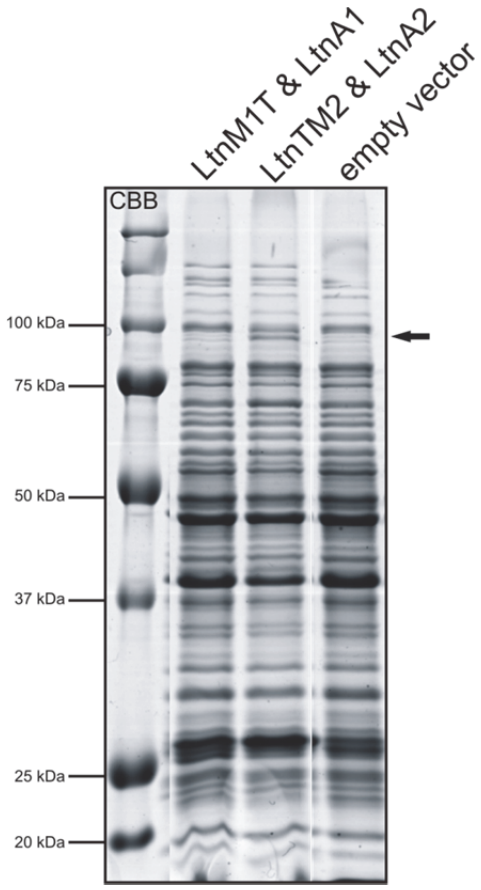

Figure 2: Coomassie stained SDS-PAGE comparing cells of *L. lactis* NZ9000 strains co-expressing LtnM1 or LtnM2 with LtnT and the corresponding substrate in comparison to a strain baring empty vectors. The arrow indicates the extra protein, LtnM2, just below 100 kDa.

Co-expression of LtnM2, LtnT and LtnA2 was described previously by Kuipers et al. (17). Plasmids, pILPTM2 and pA24, were used that expresses the aforementioned genes in *L. lactis* NZ9000. Analysis of the cells, co-expressing LtnM2, LtnT and LtnA2, on SDS-PAGE showed a clear extra protein just below 100 kDa (figure 2) not visible in the sample expressing empty vectors. The theoretical mass of LtnM2 is 106,377 Da suggesting this extra band is LtnM2. The protein runs slightly lower than its calculated mass, this can be explained by incomplete denaturation of the protein. Analysis of the culture supernatant by means of MALDI-TOF mass spectrometry yielded a peptide with a mass of 2844.24 Da (figure 3B), not found with the control strain. This mass corresponds to the theoretical mass of eight times dehydrated LtnA2, i.e., 2842.28 Da (21), suggesting dehydrated and processed LtnA2 was produced. These findings are in agreement with the results of Kuipers et al. (17).

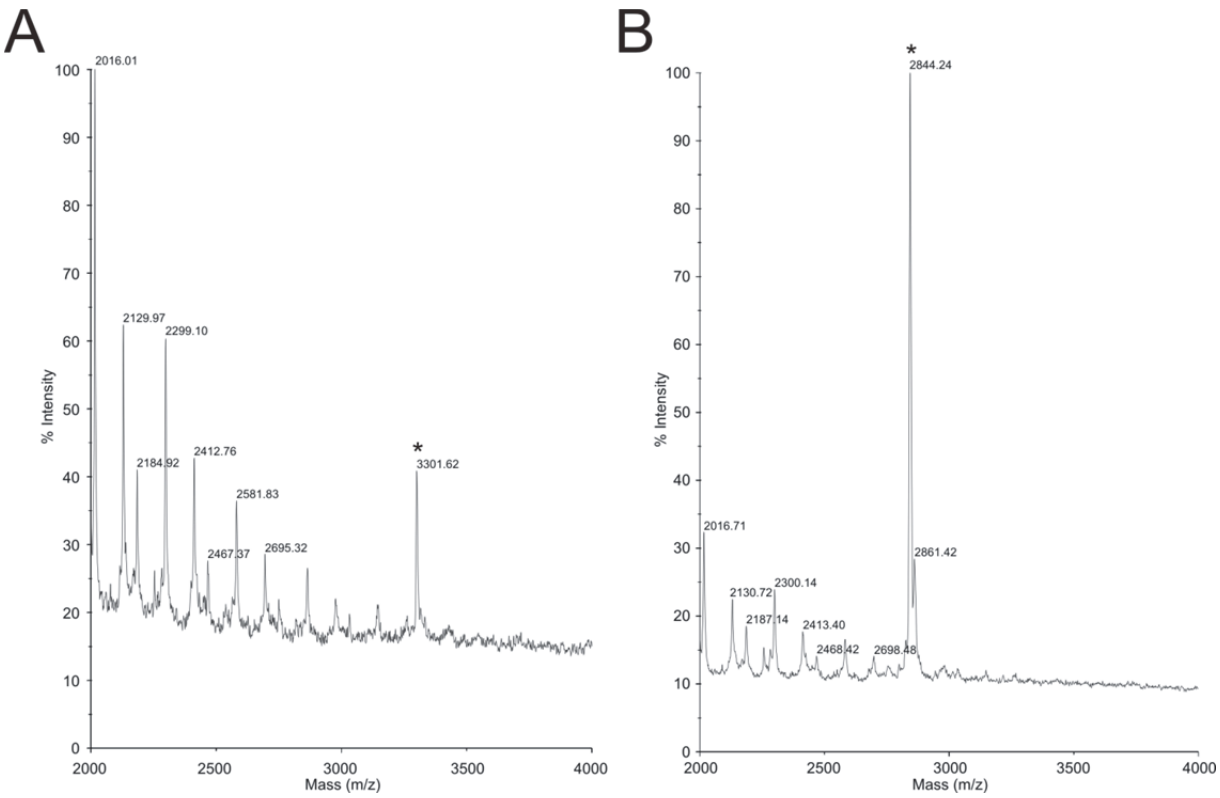

Figure 3: MALDI-TOF mass spectra of culture media showing the presence of (A) seven times dehydrated LtnA1 core peptide at 3301.62 Da (theoretical mass 3304.74 Da) after co-expression of LtnA1, LtnM1 and LtnT in *L. lactis* NZ9000, (B) eight times dehydrated LtnA2 core peptide at 2844.24 Da (theoretical mass 2842.28 Da) after co-expression of LtnA2, LtnM2 and LtnT in *L. lactis* NZ9000.

Activity of separately produced LtnA1 and LtnA2. Since lacticin 3147 is a two-component lantibiotic, antimicrobial activity is only observed when LtnA1 and LtnA2 are combined. To further assess if the separately produced LtnA1 and LtnA2 yield an active two-component lantibiotic, the individual supernatant fractions were concentrated by TCA precipitation and resuspended in MilliQ water. A dilution series of LtnA1 and LtnA2 was made using the 96-wells format and the antimicrobial activity was tested against two indicator strains, *L. lactis* LL108(pORI 280) and *L. lactis* subsp. *lactis* IL1403. As expected, the individual LtnA1 or LtnA2 peptide containing fractions did not show any activity (figure 4) as the relative growth was at the same level as the negative controls. When the LtnA1 and LtnA2 fractions were combined substantial activity against both indicator strains was observed (figure 4) consistent with the strict requirement for both peptides for bioactivity (21). When the sample is diluted up to 8-times the growth of the indicator strains is strongly inhibited and stayed below zero. After diluting the sample 16-times the indicator strains slightly regain their ability to grow, after 32-times diluting the sample the growth is still mildly inhibited compared to the negative controls, while after 64-times diluting the sample the concentration of the peptides is too low to inhibit growth. The individually produced LtnA1 and LtnA2 are about four times less active compared to natively produced lacticin 3147, that can be diluted up to 64-times before the indicator strains regain their ability to grow. In addition to the microtiter-plate assay the bioactivity was also tested in a well-diffusion assay. Concentrated TCA precipitated supernatant samples were added to a well in a plate containing the indicator strains. When LtnA1 or LtnA2 were tested alone no inhibition zone was seen, whereas combining both samples, a clear inhibition zone was evident (figure 5). These findings demonstrate that the separately produced LtnA1 and LtnA2, only modified by LtnM1 or LtnM2, respectively, are active.

Discussion

This study explored the potential of LtnM1 and LtnT for the production of class II lanthipeptides. These enzymes are normally involved in the modification, transport and maturation of LtnA1, which is a subunit of the two-component lanthipeptide lacticin 3147. LtnA1 resembles the globular shape of mersacidin, a typical class II lantibiotic (21). First it was examined if this system can be used

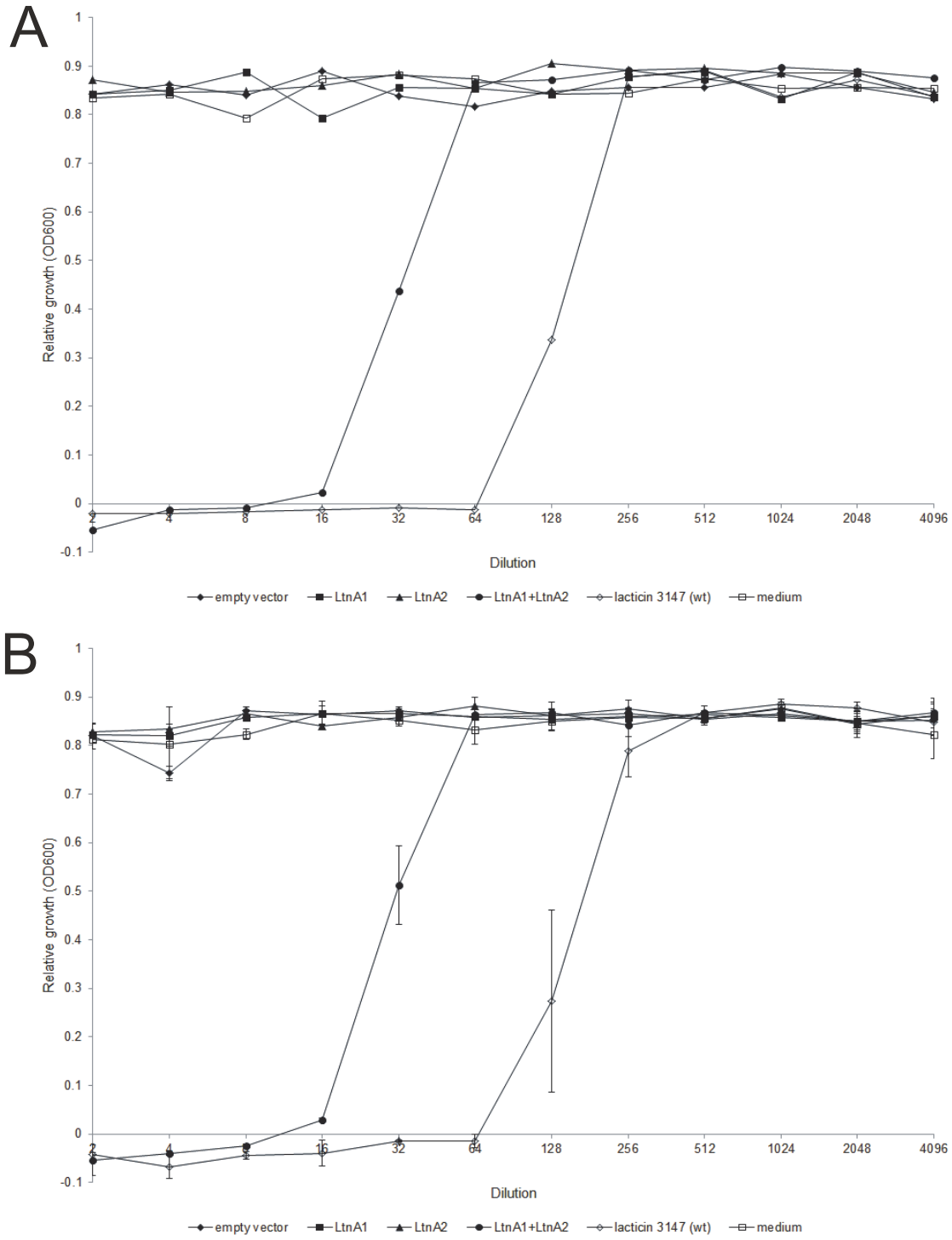

Figure 4: Microtiter plate activity assay of the activity of LtnA1 and LtnA2 against *Lactococcus lactis* LL108(pORI 280) (A) and *L. lactis* subsp. *lactis* IL1403 (B). The indicator strain was incubated for five hours with TCA precipitated supernatant of strains expressing, LtnA1, LtnM1 and LtnT (closed squares), LtnA2, LtnM2 and LtnT (closed triangle) or an 1:1 ratio of the two peptide containing samples (closed dot). The graph clearly shows that the sample containing both peptides inhibits growth of the indicator strains. As a comparison TCA precipitated supernatant of a lacticin 3147 expressing strain *L. lactis* IFPL105 (open diamond) was also included. As negative controls TCA precipitated supernatant of a strain expressing empty vectors (closed diamond) and TCA precipitated growth medium (open squares) was included.

to produce bioactive LtnA1 when expressed in a *L. lactis* host strain that normally does not produce this peptide. The first step in the formation of a (methyl)lanthionine bond is the dehydration of serine and threonine, since each dehydration reaction causes a weight loss of 18.02 Da, due to the removal of a water molecule, MALDI-TOF mass spectrometry can easily be used to make a distinction between dehydrated and non-dehydrated peptides. In addition, MALDI-TOF mass spectrometry was used to detect if the leader peptide was removed by the lantibiotic transporter. Indeed by mass spectrometry, the fully dehydrated and processed LtnA1 peptide could be detected in the culture supernatant of the expressing strain suggesting that LtnM1 and LtnT are indeed able to modify, process and transport LtnA1.

Since the second step in the (methyl)lanthionine formation, the cyclisation via a thioether bond with a free cysteine, does not cause any changes in mass, the ring formation cannot be determined by MALDI-TOF mass spectrometry

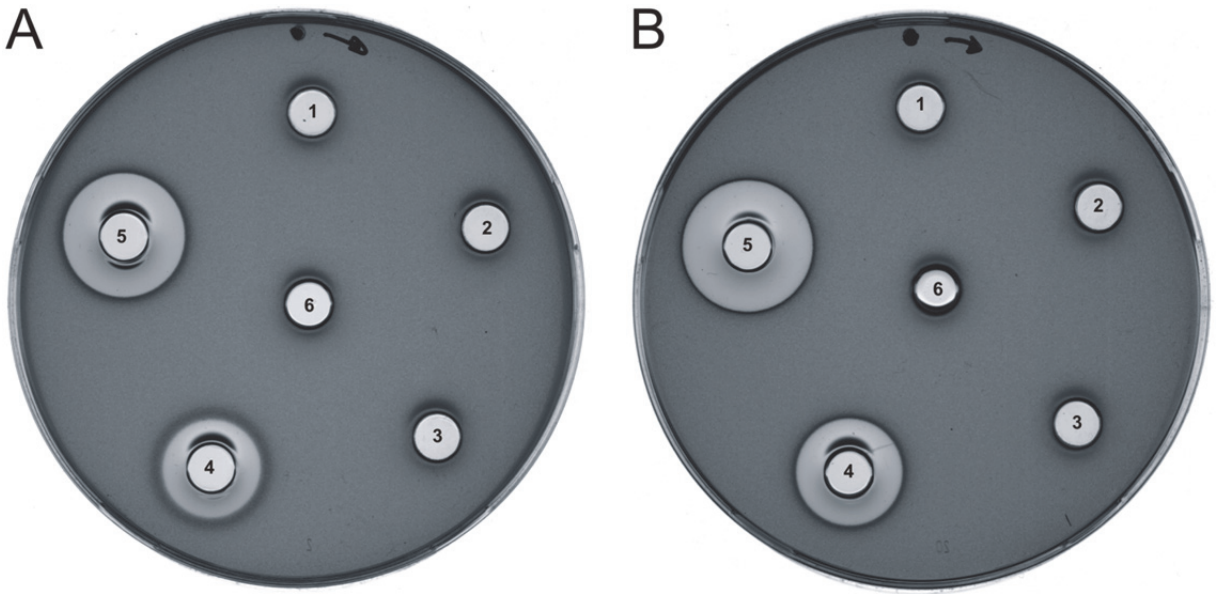

Figure 5: Activity assay of LtnA1 and LtnA2 against *Lactococcus lactis* LL108(pORI 280) (A) and *L. lactis* subsp. *lactis* IL1403 (B). The indicator strain was incubated with 4x concentrated TCA precipitated supernatant of strains expressing, LtnA1, LtnM1 and LtnT (2), LtnA2, LtnM2 and LtnT (3) or an 1:1 ratio of the two peptide containing samples (4). The halo formed at the sample containing both peptides clearly shows that LtnA1 and LtnA2 inhibit growth of the indicator strains. As a comparison 4x concentrated TCA precipitated supernatant of the lacticin 3147 expressing strain *L. lactis* IFPL105 (5) was also included. As negative controls 4x concentrated TCA precipitated supernatant of a strain expressing empty vectors (1) and TCA precipitated growth medium (6) was included.

solely without further modification and fragmentation. Without the full modification, dehydration and cyclization, the lantibiotic is not active, therefor the activity assays were used to determine if the separately produced LtnA1 and LtnA2 were fully modified and thereby active. The results show that combining LtnA1 and LtnA2 they are able to inhibit growth, concluding that they are active and the (methyl)lanthionine rings are formed. Interestingly, activity was obtained despite the fact that the additional modification, the conversion of L-serine to D-alanine, by LtnJ was not present. It was previously reported that in the absence of LtnJ, a major reduction in activity is observed (22). Figure 4 shows that the activity of the two separate produced peptides is about four times lower than the activity of the wild type produced lacticin 3147. This phenomenon could be due to a low production level and/or the absence of the LtnJ modification.

As described before for other lantibiotic modifying enzymes like LtnM2 (17), NukM (23) and ProcM (24), LtnM1 is able to modify its natural substrate without the presence of other lantibiotic enzymes encoded in the lantibiotic gene cluster. Therefore LtnM1 and LtnM2 could form interesting targets to be used in bioengineering studies to produce novel lanthipeptides from diverse sources.

Acknowledgments

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Table 2: Peptide sequence of the expressed (fusion) peptides and their theoretical mass.

	Lantibiotic leader peptide	Lantibiotic core peptide	Mass (Da) core peptide*	Mass (Da) LtnM1/LtnM2 modified core peptide*
	-25 -20 -15 -10 -5 -1	1 5 10 15 20 25 30		
LtnA1	MNKNEIETQPVTWLEEVSDQNFDEDVFGA	CSTNTFSLSDYWGNNGAWCTLTHECMAWCK	3430.88 (21)	3304.74 (21)
	-35 -30 -25 -20 -15 -10 -5 -1	1 5 10 15 20 25		
LtnA2	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG	TTPATPAISILSAYTSTNTCPTTKCTRAC	2986.44 (21)	2842.28 (21)

* The monoisotopic mass of the peptides $(M+H)^+$
TS Ser or Thr residues that are dehydrated in wild type situation
C Cys residues that form a (Me)Lan-ring in wild type situation

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Chapter 4

Assessing the specificity of the lacticin 3147 modifying enzymes and transporter as a generic production system for class II lantibiotics

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Abstract

Class II lantibiotics are post-translationally modified by a multifunctional enzyme, LanM, that catalyzes the dehydration of serine and threonine residues as well as thioether bridge formation between these residues and cysteine. To examine the promiscuity of the LanM enzyme and the associated transporter LanT for the production of class II lantibiotics, the two-component lantibiotic lacticin 3147 was studied. The active lantibiotic consists of two structural lanthipeptides, LtnA1 and LtnA2 that are modified by LtnM1 and LtnM2, respectively. A set of variants of LtnA1 and LtnA2 were created that include swapped leader peptides, cysteine to alanine mutants to prevent thioether bridge formation and the introduction of factor Xa protease site in LtnA1. The data suggest a high specificity of the LtnM1 and LtnM2 systems for their cognate substrates, however LtnM2 also allowed production of a Cys-less variant of LtnA1 when expressed with the leader peptide of LtnA2.

Introduction

Resistance is currently a serious threat during treatment of infectious disease. For many of the currently used antibiotics, resistance mechanisms have developed that spread among microbes. Therefore, there is an urgent need for a new generation of antibiotics based on different chemical structures than those that have been employed so far. Lantibiotics are an interesting group of antimicrobial peptides with a potential use as antibiotics. Lantibiotics belong to a larger group of peptides called lanthipeptides. Lanthipeptides are ribosomal synthesized peptides that contain the unusual amino acids meso-lantihionine (Lan) and (2S,3S,6R)-3-methyllanthionine (MeLan). These amino acids are formed during post-translational modification in which serine and threonine residues are dehydrated to form a 2,3-dehydroalanine or (Z)-2,3-dehydrobutyrine, respectively. The formed dehydroalanine or dehydrobutyrine residues are then coupled to a cysteine via a thioether bond to form the lanthionine or methyllanthionine rings, respectively. Based on the modification enzymes that introduce the (methyl)lanthionine residues, lanthipeptides can be divided into four classes (1). Class I and II lanthipeptides are mainly comprised of the lantibiotics, lanthionine-containing antibiotics. In class I lanthipeptides/lantibiotics, the formation of a lanthionine ring is catalyzed by a dehydratase (LanB) and a cyclase (LanC), whereas in class II lantibiotics both reactions are catalyzed by a single bifunctional enzyme, LanM. LanM enzymes typically contain a N-terminal dehydratase domain, which does not show any homology to LanB. The C-terminal domain of LanM contains a cyclase domain which has ~25% sequence identity to LanC (1), including the conserved zinc-binding residues that are necessary for NisC catalysis (2). Class III lanthipeptides are modified by a multifunctional synthetase (LanKC) containing a N-terminal lyase domain, a central kinase domain and a putative cyclase domain at the C-terminus (3). Class IV lanthipeptides are also modified by a trifunctional enzyme (LanL), with a similar domain organization as found for class III lanthipeptides except that the cyclase domain is homologous to LanC (4). Besides the difference in the C-terminal cyclase domain, LanKC enzymes can also generate labionin structures, formed from two dehydroalanines and a cysteine residue, while LanL enzymes only generate lanthionine rings.

The genes involved in lantibiotic biosynthesis are usually found in a cluster that harbors the gene for the structural peptide (LanA) and the modification enzymes (e.g. LanBCM). Lantibiotics may undergo further post-translational modifications like decarboxylation (LanD) or the conversion of a L-serine into a D-alanine (LanJ). In addition, the clusters encode genes specifying for an ATP-binding cassette (ABC) transporter (LanT) to excrete the peptide over the membrane, a protease to cleave of the leader sequence (LanP), genes encoding proteins for self-protection/immunity (LanIFEG) and genes encoding the regulation of lantibiotic production (LanKR). The gene, *lanA*, encodes the linear precursor peptide. The N-terminal part of LanA contains a leader peptide that is recognized by the modification enzymes and transporter, the C-terminus of LanA encodes the core peptide. The core peptide is modified and matures in its final active form after leader peptide removal. With class I lantibiotics, maturation occurs extracellular by the protease LanP. However with class II lantibiotics a bifunctional transporter is responsible for both the maturation and secretion. This transporter harbors an intracellular protease domain, thus maturation likely precedes excretion. The leader peptide of class II lantibiotics contains an ELxxBx motif (B=V, L, I) which usually ends in the double glycine motif, GG, GA or GS/T, which distinguishes it from the leader peptide of class I lantibiotics and it is believed that this double glycine motif is recognized by the N-terminal peptidase C39 domain of the bifunctional transporter. The ELxxBx motif is generally not found in the two-component lantibiotics like lacticin 3147, nonetheless they do contain the distinctive double glycine motif (5).

Lantibiotics form an interesting group of peptides that may be used as potential new antibiotics. Since lantibiotics are gene encoded and ribosomally synthesized they form an advantage over non-ribosomal or polyketide antibiotics in bioengineering applications (6). Moreover, until now no known resistance against lantibiotics is reported, an example is the lantibiotic nisin that is already for over 50 years used as a food preservative without significant development of microbial resistance. The antimicrobial activity of only a few lantibiotics has been studied extensively, and believed to emerge from inhibiting cell wall biosynthesis, disruption of the membrane integrity by forming pores, or by a combination of the two mechanisms (1). Lantibiotics are only active against Gram-positive bacteria as these peptides cannot pass the outer membrane of Gram-negative bacteria. A less desirable property of some lantibiotics is that they can be

susceptible to proteolytic degradation (8) and their solubility can also be limited (9). In addition, the presence of dehydrolanines can render the peptide unstable unless the pH is low. However this property can be reversed in many cases by removing dehydroalanines by mutagenesis without the loss of activity.

Lanthipeptides are mainly produced by Gram-positive bacteria like, *Lactococcus*, *Bacillus*, *Staphylococcus*, *Streptococcus*, and by some *Streptomyces* species (10, 11). By genome-mining, many genes associated with lanthipeptides have been identified. This has expanded the distribution of lanthipeptides also to other firmicutes, actionomycetes, bacteroidetes, chlamydiae, proteobacteria and cyanobacteria (4, 12-14). The production of these lanthipeptides by their natural host is complicated as many of these organisms have not been cultured nor genetically characterized while also the expressing conditions are unknown. A potential solution for this problem is the development of a heterologous expression system to produce new/putative lanthipeptides. Such a system has been described for class I lanthipeptides based on the modification enzymes (NisBC) and transporter (NisT) of nisin (15-19). These enzymes turned out to be sufficiently relaxed in their substrate specificity to also modify and secrete unrelated lanthipeptides. However, such a generic system has not yet been reported for class II lanthipeptides.

It was shown before that LtnM2 and LtnT, that specify the modifying enzyme and transporter of one of the structural lanthipeptides of the two-component lantibiotic lacticin 3147 (figure 1), can modify, process and transport LtnA2 and unrelated peptides such as nisin and angiotensin variants that are fused behind the lacticin A2 leader (20). Also, LtnM1 and LtnM2 when individually co-expressed with the transporter LtnT have been shown to modify, process and secrete their natural substrates, LtnA1 and LtnA2, respectively (Chapter 3). When the individually produced LtnA1 and LtnA2 are combined, an active lacticin 3147 can be reconstituted. These findings suggest the biosynthesis enzymes of lacticin 3147 have the potential to form the basis of a heterologous expression system for class II lanthipeptides. This study has examined the specificity of LtnM1 and LtnM2 towards potential substrates and explored the possibility to use these enzymes for the development of such a generic class II lanthipeptide production system.

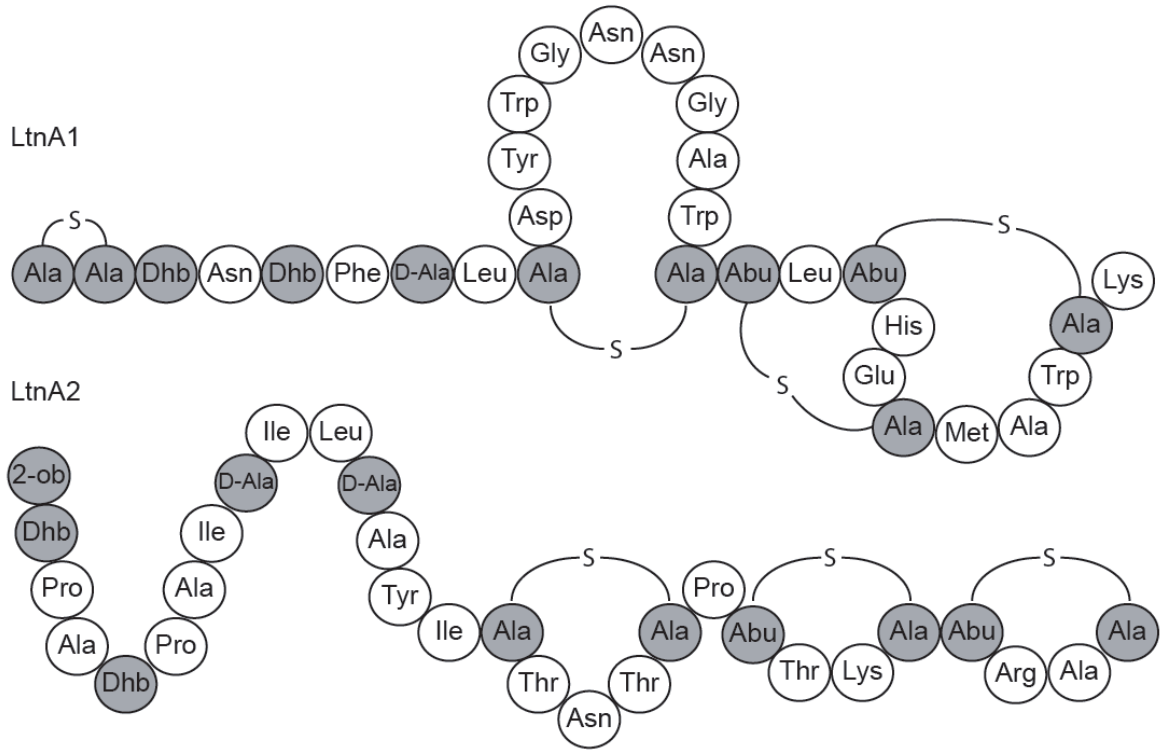

Figure 1: Shorthand notation of mature LtnA1 and LtnA2 of the two-component lantibiotic lacticin 3147 (after the example from Cotter et al. (34)). The modified residues are marked in gray.

Material and Methods

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in table 1. *Lactococcus lactis* NZ9000 was used as a host for the plasmids. All strains were grown at 30°C in Bacto M17 broth (Becton Dickinson Difco) supplemented with 0.5% glucose. Where needed chloramphenicol (5 µg/mL), erythromycin (5 µg/mL) or both antibiotics (3 µg/mL) were added to the medium. *Escherichia coli* was cultured at 37°C in LB Broth (Lennox) (Carl Roth) and supplemented with ampicillin (50 µg/mL).

Molecular cloning and plasmid construction. Plasmid construction was carried out using standard genetic methods (21). For PCR, Phusion High-Fidelity DNA Polymerase was used and all restriction and DNA modifying enzymes were purchased from Thermo Scientific. All constructs were validated by sequence

analysis performed at Macrogen Europe. PCR primers used in this study are listed in table 2.

Table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
Lactococcus lactis NZ9000	MG1363 derivative; pepN::nisRK+	(23)
Escherichia coli DH5α		
L. lactis LL108 (pORI 208)	Em^r Cm^r	(23, 24)
L. lactis IFPL105	pBAC105, lacticin 3147 producing strain	(32)
L. lactis subsp. *lactis* IL1403		
Plasmids		
pIL253	Em^r	(33)
pILPltnT-2	pIL253 derived; *ltnT-ltnM2* cloned behind the *Pnis* promoter, disruption of ltnM2 by BglII digestion; Em^r	Kind gift from Lanthio Pharma. (20)
pILPM1T	pIL253 derived; *ltnM1-ltnT* cloned behind the *Pnis* promoter; Em^r	Kind gift from Lanthio Pharma
pILPTM2	pIL253 derived; *ltnT-ltnM2* cloned behind the *Pnis* promoter; Em^r	Kind gift from Lanthio Pharma. (20)
pNZ8048	Cm^r	(23)
pA13	pNZ8048 derived; *ltnA1* cloned behind the *Pnis* promoter; Cm^r	Kind gift from Lanthio Pharma
pA24	pNZ8048 derived; *ltnA2* cloned behind the *Pnis* promoter; Cm^r	(20)
pMA38	Plasmid containing synthetic DNA of leader peptide LtnA1(-29 to -1)::LtnA2(1 to 29) behind the *Pnis* promoter, BglII and HindIII restriction sites, Amp^r	Mr. Gene GmbH
pMA39	Plasmid containing synthetic DNA of leader peptide LtnA2(-36 to -1)::LtnA1(1 to 30) behind the *Pnis* promoter, BglII and HindIII restriction sites, Amp^r	Mr. Gene GmbH
pA38	pNZ8048 derived; leader peptide LtnA1(-29 to -1)::LtnA2(1 to 29) behind the *Pnis* promoter; Cm^r	This study
pA39	pNZ8048 derived; leader peptide LtnA2(-36 to -1)::LtnA1(1 to 30) behind the *Pnis* promoter; Cm^r	This study
pA13-xa1	pNZ8048 derived; leader peptide LtnA1(-29 to -5)::IDGR::LtnA1(1 to 30) behind the *Pnis* promoter; Cm^r	This study
pA13-xa2	pNZ8048 derived; leader peptide LtnA1(-29 to -1)::IDGR::LtnA1(1 to 30) behind the *Pnis* promoter; Cm^r	This study
pNZ-A62	pNZ8048 derived; LtnA1(C-less) behind the *Pnis* promoter; Cm^r	This study
pNZ-A72	pNZ8048 derived; LtnA2(C-less) behind the *Pnis* promoter; Cm^r	This study
pNZ-A82	pNZ8048 derived; leader peptide LtnA1(-29 to -1)::LtnA2(1 to 29)(C-less) behind the *Pnis* promoter; Cm^r	This study
pNZ-A92	pNZ8048 derived; leader peptide LtnA2(-36 to -1)::LtnA1(1 to 30)(C-less) behind the *Pnis* promoter; Cm^r	This study

Em^r erythromycin resistance marker
Cm^r chloramphenicol resistance marker
Amp^r ampicilin resistance marker

Plasmid pA13 (kind gift form Lanthio Pharma) is derived from pNZ8048 and contains the *ltnA1* gene cloned behind the *Pnis* promoter. This plasmid was used to create pA13-xa by mutagenesis PCR wherein the last four amino acids (VFGA) of the LtnA1 leader sequence were replaced with the factor Xa protease site (IDGR). Likewise, pA13-xa2 was generated by a mutagenesis PCR inserting the factor Xa protease site in between the LtnA1 leader sequence and core peptide. To determine if the factor Xa protease site encoding sequence was inserted correct a sequence analysis was performed by Macrogen Europe.

pA38 and pA39 were created by replacing the *Pnis* promotor and the LtnA1 encoding sequence of pA13 using the BglII and HindIII restriction sites by the chimeric products containing the *Pnis* promoter followed by LtnA1(-29 to -1)::LtnA2(1 to 28) or LtnA2(-36 to -1)::LtnA1(1 to 30) using synthetic fragments of DNA that were obtained from Mr. Gene GmbH.

For the creation of pNZ-A62, pNZ-A72, pNZ-A82 and pNZ-A92, that harbor cysteine less core peptide encoding sequences, synthetic constructs were ordered at GeneArt (Life Technologies). The plasmid DNA supplied by GeneArt was used in a PCR to amplify the region containing the Cys-less peptides. The PCR-products were digested using HindIII and NcoI and subsequently ligated into HindIII and NcoI digested pNZ8048. The resulting plasmid was subjected to mutagenesis PCR to remove the extra start codon from the NcoI restriction site. All constructs were verified by DNA sequence analysis by Macrogen Europe.

Constructs were transformed to *L. lactis* NZ9000 in combination with pILPltnT-2 (20), pILPM1T (kind gift form Lanthio Pharma) or pILPTM2 (20) by means of electrotransformation as described previously by Holo et. al. (22) using a Bio-Rad gene pulser.

MALDI-TOF mass spectrometry. *L. lactis* expression strains were grown overnight in GM17 and diluted 50-fold in minimal medium, this medium was prepared as described by Rink et al. (19) or else the vitamin mix was replaced by BME Vitamins (Sigma). Cultures were grown for 3 hours at 30°C before expression was induced by adding 1 ng/mL nisin (Sigma-Aldrich). After 24 (and 4) hours of incubation, the supernatant was collected by centrifugation (20 minutes at 3724 x g). A sample of 1 µL of the supernatant was applied to the MALDI-TOF target, dried and washed with a drop of MilliQ water. Subsequently 1 µL of matrix, 5 mg/mL α-Cyano-4-hydroxycinnamic acid (Sigma-Aldrich)

dissolved in 50% Acetonitrile and 0,1% trifluoroacetic acid (TFA), was added to the sample and dried.

In addition, peptides were precipitated from the supernatant by the addition of 10% trichloroacetic acid (TCA) and incubation on ice for 30 min. Next, the sample was spun for 30 min. at 17,000 x g and the pellet was washed with acetone. To remove the acetone, the sample was spun 15 min. at 17,000 x g and dried for 5 min. at 42°C. TCA precipitated material was dissolved in 10 µL MilliQ water, and 1 µL of the sample was applied to the MALDI-TOF target, dried, washed and treated as described above.

MALDI-TOF mass spectrometry spectra were recorded with a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). The recorded spectra were analyzed with Data Explorer TM 4.0.0.0 (Applied Biosystems). An external calibration was used to correct the resulting spectra.

Table 2: Primers used in this study

Primer name	**Primers sequence (5'-3')**	**Characteristics**
001-fw	ATTGACGGCCGTTGTAGTACTAACACATTCTCGCTCAGTG	Phosphorylated 5'-end, encoding factor Xa protease site
002-rv	ATCTTCATCAAAATTTTGATCAGATACTTCTT	Phosphorylated 5'-end, reversed primer for factor Xa protease site introduction
022-rv	CGCACCAAATACATCTTCATC	reversed primer for factor Xa protease site introduction
019-fw	TAATACGACTCACTATAGGGC	Forward primer for amplifying Cys-less lantibiotic gene from synthetic construct supplied by GeneArt
020-rv	GACAGGTTTCCCGACTGGAAAG	reversed primer for amplifying Cys-less lantibiotic gene from synthetic construct supplied by GeneArt
170-rv	GGTGAGTGCCTCCTTATAATTTATTTTG	Phosphorylated 5'-end, reversed primer for removing extra start codon in Cys-Less constructs
171-fw	ATGAACAAAAATGAAATTGAAACACAAC	forward primer for removing extra start codon in Cys-Less constructs encoding Ltn1 leader peptide
172-fw	ATGAAAGAAAAAAATATGAAAAAGAATG	forward primer for removing extra start codon in Cys-Less constructs encoding Ltn2 leader peptide

ATCG sequence encoding factor Xa protease site.

Antimicrobial activity assay. *L. lactis* expression strains overnight grown in GM17 were diluted 60-fold in fresh GM17 medium and incubated at 30°C until it reached an optical density at 600 nm (OD_{600}) between 0.4-0.6. The expression was induced by adding nisin (Sigma-Aldrich) to an end concentration of 1 ng/mL. After 4 or 24 hours of incubation the cells were removed from the supernatant by centrifugation (20 min. at 3724 x g). The supernatant was subjected to TCA precipitation as described above, and the pellet was dissolved in GM17, with appropriate antibiotics, as a one-fold concentrated sample.

The concentrated TCA precipitated samples were used to create two-fold diluted series in a microtiter-plate. These samples were overlaid in an one to one ratio with an indicator strain. After 5 hours of incubation at 30°C the growth inhibition was measured by measuring the OD_{600}. The indicator strains, *L. lactis* LL108(pORI 280) (23, 24) and *L. lactis* subsp. *lactis* IL1403, were prepared as followed; an overnight culture of the indicator strain was diluted 100-fold in fresh medium and grown at 30°C until OD_{600} reached 0.2-0.3 before added to the lanthipeptide samples.

Results

Substrate promiscuity of the LtnM1 and LtnM2 systems. The secondary structure of the two individual entities of the two-component lantibiotic lacticin 3147 differ. The mature LtnA1 resembles the globular shape of mersacidin, a typical class II lantibiotic, while LtnA2 resembles the elongated shape of nisin, a class I lantibiotic (25). Previously, it was shown that co-expression of LtnM1 and LtnT in *L. lactis* NZ9000, results in the complete modification and export of LtnA1. In addition, co-expression of LtnM2 and LtnT support the modification and export of LtnA2. When these two peptides are combined, an active antimicrobial lacticin 3147 is obtained (Chapter 3). However, it is unknown if LtnM1 can modify LtnA2 or reversely, if LtnM2 can modify LtnA1. For this purpose LtnA2 was co-expressed with LtnM1/LtnT in *L. lactis* NZ9000, and the supernatant of the expressing strain was analyzed by MALDI-TOF mass spectrometry for the presence of mature LtnA2. Compared to a control bearing the empty vectors, no masses could be found that can be attributed to LtnA2. Likewise, when LtnA1 was co-expressed with LtnM2/LtnT, no production of LtnA1

Table 3: Peptide sequence of the expressed (fusion) peptides and their theoretical mass.

	Lantibiotic leader peptide	Factor Xa protease site	Lantibiotic core peptide	Mass (Da) precursor peptide*	Mass (Da) core peptide**
Wild type peptides					
	-25 -20 -15 -10 -5 -1		1 5 10 15 20 25 30		
LtnA1	MNKNEIETQPVTWLEEVSDQNFDEDVFGA		CSTNTFSLSDYWGNNGAWCTLTHECMAWCK	6668.24	3430.88(25)
	-35 -30 -25 -20 -15 -10 -5 -1		1 5 10 15 20 25		
LtnA2	MKEKNMKKNDTTELQLGKYLEDDMIELAEGDESHGG		TTPATPAISILSAYISTNTCPTTKCTRAC	6949.84	2986.44(25)
Fusion peptides					
LtnA1-A2	MNKNEIETQPVTWLEEVSDQNFDEDVFGA		TTPATPAISILSAYISTNTCPTTKCTRAC	6224.87	2986.44
LtnA2-A1	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG		CSTNTFSLSDYWGNNGAWCTLTHECMAWCK	7393.21	3430.88
Cysteine less peptides					
LtnA1(C-less)	MNKNEIETQPVTWLEEVSDQNFDEDVFGA		ASTNTFSLSDYWGNNGAWATLTHEAMAWAK	6539.99	3301.50
LtnA2(C-less)	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG		TTPATPAISILSAYISTNTAPTTKATRAA	6853.66	2890.55
LtnA1-A2(C-less)	MNKNEIETQPVTWLEEVSDQNFDEDVFGA		TTPATPAISILSAYISTNTAPTTKATRAA	6128.69	2890.55
LtnA2-A1(C-less)	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG		ASTNTFSLSDYWGNNGAWATLTHEAMAWAK	7264.97	3301.50
Factor Xa containing peptides					
LtnA1-xa1	MNKNEIETQPVTWLEEVSDQNFDED	IDGR	CSTNTFSLSDYWGNNGAWCTLTHECMAWCK	6735.29	n/a
LtnA1-xa2	MNKNEIETQPVTWLEEVSDQNFDEDVFGA	IDGR	CSTNTFSLSDYWGNNGAWCTLTHECMAWCK	7109.73	3870.62

* The average mass of the proteins, $(M+H)^+$, was calculated without the initial methionine.
** The monoisotopic mass of the peptides $(M+H)^+$
TS Ser or Thr residues that are dehydrated in wild type situation
C Cys residues that form a (Me)Lan-ring in wild type situation

could be demonstrated. This suggests that LtnM1 and LtnM2 are equipped with a high degree of substrate specificity.

Possibly, the apparent substrate specificity arises from a defective in the targeting of the structural peptides to the LtnM modifying enzymes as the leader peptides of LtnA1 and LtnA2 differ in amino acid sequence (table 3). Both leader peptides are typical for class II lanthipeptides as they contain the double glycine motif. Therefore, the leader peptides of LtnA1 and LtnA2 were swapped creating peptides containing the LtnA2 core peptide fused behind the LtnA1 leader peptide (LtnA1-A2) and a peptide containing the LtnA1 core peptide fused behind the LtnA2 leader peptide (LtnA2-A1) (for peptide sequence see table 3). The fusion peptides were co-expressed with LtnM1/LtnT and LtnM2/LtnT, and concentrated supernatant of the expression cultures was analyzed by MALDI-TOF mass spectrometry for the presence of the unprocessed and/or mature peptides. In none of the combinations peptides could be detected. Also, bioactivity assays did not reveal the presence of active peptides. This further suggests a high substrate specificity of LtnM1 and LtnM2 being active towards their own structural lanthipeptide only.

Substrate promiscuity of the LtnT transporter. The lacticin 3147 transporter, LtnT, transports both LtnA1 and LtnA2 across the membrane. This implies a less stringent substrate specificity as compared to the modifying enzymes. To test if LtnT can process and transport the fusion peptides, they were co-expressed in *L. lactis* NZ9000 without the cognate LtnM enzymes. This yielded no peptide products suggesting that LtnT may only secrete the modified peptides or that there might be a functional link between LtnT and the modifying enzymes. To examine this phenomenon in more detail, the expression studies were performed in the presence of the respective LtnM enzymes but to prevent full modification, all cysteine residues in the core peptides of LtnA1 and LtnA2 were replaced by alanine residues, LtnA1(C-less) and LtnA2(C-less) (table 3). Thus, in these peptides it is no longer possible to introduce thioether bridges albeit dehydration can still occur. First it was examined if LtnT is able to export the Cys-less LtnA1 and LtnA2 by co-expression in *L. lactis* NZ9000. However, no peptide products could be detected by MALDI-TOF mass spectrometry in the case of Cys-less LtnA2, while with Cys-less LtnA1, a specific mass was detected at

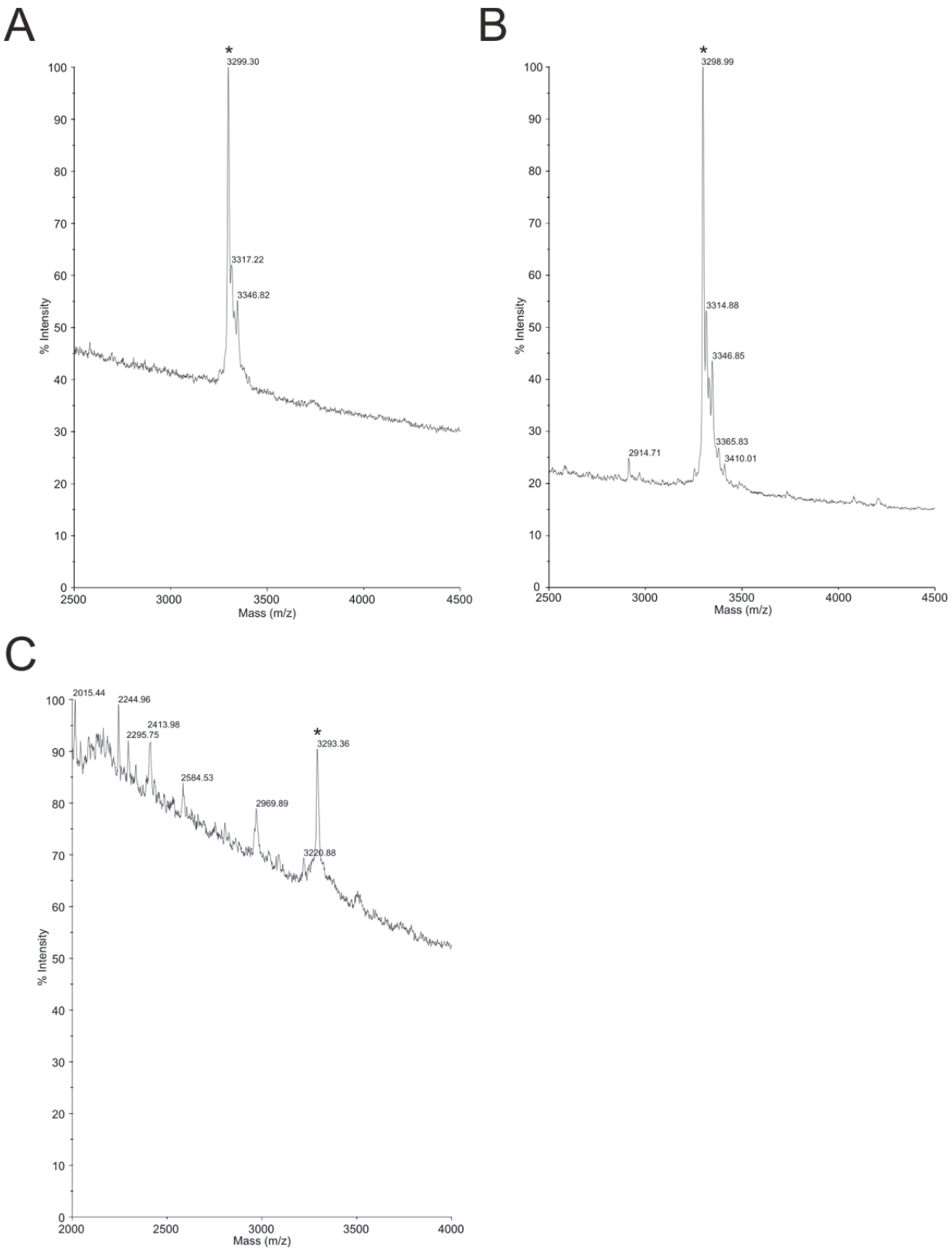

Figure 2: MALDI-TOF mass spectra of culture media showing the presence of (A) unmodified Cys-less LtnA1 core peptide at 3299.30 Da after co-expression of LtnA1(C-less) and LtnT in *L. lactis* NZ9000, (B) unmodified Cys-less LtnA1 core peptide at 3298.99 Da after co-expression of fusion peptide LtnA2-A1(C-less) and LtnT in *L. lactis* NZ9000, (C) unmodified Cys-less LtnA1 core peptide at 3293.36 Da after co-expression of LtnA2-A1(C-less), LtnM2 and LtnT in *L. lactis* NZ9000.

3299.30 Da (figure 2A) that was not present in the supernatant of the control strain that bears the empty vectors. This mass corresponds closely to that of the unmodified but processed Cys-less LtnA1 with a predicted mass of 3301.50 Da. In addition, in the supernatant of a strain co-expressing LtnT and the chimeric Cys-less LtnA1-A2, no peptides were found. Although, in the supernatant of a strain co-expressing LtnT and the chimeric Cys-less LtnA2-A1, a mass of 3298.99 Da was detected (figure 2B) that corresponds to unmodified and processed Cys-less LtnA1 core peptide. These results suggest that LtnT is able to transport and process Cys-less LtnA1 even when fused behind the leader peptide of LtnA2, and that there is no functional link with LtnM1 per se.

Next, the supernatant of *L. lactis* NZ9000 strains co-expressing LtnM1/LtnT in combination with LtnA1(C-less) and LtnA1-A2(C-less) were analyzed by MALDI-TOF mass spectrometry. None of these conditions yielded any of the expected peptides. Also, co-expression of LtnM2/LtnT with LtnA2(C-less), did not give rise to produced peptides whereas LtnA2-A1(C-less) expression yielded a product with a unique mass of 3293.36 Da (Figure 2C), which matches the theoretical mass of non-dehydrated but processed Cys-less LtnA1, 3301.50 Da as described above. These results indicate that LtnM2/LtnT is not able to modify Cys-less LtnA1 but that it is able to process and transport the Cys-less peptide behind the leader peptide of LtnA2. Apparently, part of the specificity of LtnM2 is leader peptide dependent.

Modification of the LtnA1 leader peptide. The above system allows for the production of an active and maturated lantibiotic as LtnT removes the leader peptide during transport. However, for the production of uncharacterized lanthipeptides it will be necessary to generate an inactive form that in a further processing step can be activated to the mature lanthipeptide as otherwise growth of the production strain might be inhibited. A possible strategy to achieve this goal is to replace the double glycine motif of the leader peptide with a specific protease site so the leader peptide can be removed in vitro. With nisin, a factor Xa site could be introduced in between the leader and core peptide without disturbing modification by NisB/NisC and transport by NisT (26). Therefore, a factor Xa protease site (IDGR) was placed between the leader and core peptide of LtnA1 and in another approach, the last four amino acids of the leader peptide were replace by the same cleavage site (for peptide sequence see table 3). The

plasmids encoding the cleavage site containing peptides were co-expressed with LtnM1/LtnT. When the supernatant was screened with MALDI-TOF MS no peptides were detected nor could antimicrobial activity be detected in a bioactivity assay where the supernatant fraction was combined with LtnA2. These results suggest that the LtnM1/LtnT system cannot accommodate the production of cleavage site modified LtnA1 peptides.

To assess if LtnT alone is able to export the cleavage site modified LtnA1 peptides, LtnT was co-expressed with either LtnA1-xa1 or LtnA1-xa2 in *L. lactis* NZ9000. After co-expression supernatant of the expression cultures was examined by MALDI-TOF mass spectrometry for the presence of unprocessed LtnA1-xa1 or LtnA1-xa2. However, no peptides were detected. These results suggest that the presence of the double glycine motif in the leader peptide is important for the transport activity by the lacticin 3147 transporter.

Discussion

Novel antibiotic substances are necessary in the battle against multi drug resistances. For many antibiotics of biological origin, it is difficult to modify them as their production usually is a multi-enzymatic processes. Lantibiotics are ribosomal synthesized and gene-encoded and therefore more susceptible to bioengineering. Bioengineering with biosynthesis enzymes of lantibiotics could give rise to a novel antibiotic peptide. For that reason, lanthipeptide modification enzymes with broad substrate specificity need to be found. Previously, it was shown that LtnT is able to process and translocate LtnA1 and LtnA2 (Chapter 2). When introducing LtnM1 or LtnM2 to the production system, modified processed and active LtnA1 or LtnA2 is produced, respectively (Chapter 3). In this study, the focus was on determining if LtnM1, LtnM2 and LtnT can be possible candidates to be used in a generic class II lanthipeptide production system. The next question was to determine if LtnA1 and LtnA2 could be modified by their counterparts modification enzyme since lacticin 3147 has two distinct modification enzymes for each of these substrates (27). However, no peptides related to LtnA1 or LtnA2 were observed by MALDI-TOF mass spectrometry analysis. These findings suggest that the modification enzymes are highly specific, but alternatively, this specificity may relate only to the targeting or activation of LtnM as the two peptides bear different leader peptides. In addition,

the absence of peptides in the supernatant suggests that the presence of the modification enzymes prevent the export of the peptides since previous results show that the transporter is able to export and process the unmodified peptides (Chapter 2). Furthermore, these data suggest a functional link between the modification enzymes and the transporter.

To investigate the substrate specificity of LtnM1 and LtnM2 system further, the leader peptides of LtnA1 and LtnA2 were swapped and tested for production. However, in this case no peptides could be detected. This suggests a high specificity of the LtnM1 and LtnM2 enzymes. Previously, it was shown that LtnM2 is able to modify LtnA2 unrelated peptides when fused behind the LtnA2 leader peptide (20). LtnA1 resembles the globular structure of mersacidin and LtnA2 resembles the elongated structure of nisin (25). Possibly, the respective LtnM enzymes can only handle core peptides with a similar overall structure. To examine this phenomenon in further detail, we also expressed variants of LtnA1 and LtnA2 lacking all cysteine residues. Such peptides can still be dehydrated but thioether linkages can no longer be formed. Thus, such peptides are essentially unstructured. Also, this did not lead to the production of (high levels) of peptide except when LtnT or LtnM2/LtnT was co-expressed with a LtnA2-A1(C-less). This yielded a processed but unmodified Cys-less variant of LtnA1. This observation is in line with the results of Kuipers et.al. (20) who showed that LtnM2 is able to modify LtnA2 unrelated peptides when fused to the LtnA2 leader peptide. However, it is unclear why LtnM2/LtnT did not produce the Cys-Less LtnA2 fused to its native leader peptide. In addition co-expression of LtnT with Cys-less LtnA1 revealed the presence of the processed Cys-less LtnA1 core peptide. The fact that not all dehydrated residues in the wild type lacticin 3147, namely Thr3 and Thr5 of LtnA1 and Thr2 and Thr5 of LtnA2 (figure 1), are used in a next modification suggest that the modification enzymes are also able to only dehydrate residues. Since LtnT was able to process and export Cys-less LtnA1, with LtnM1 a processed and at least two times dehydrated Cys-less LtnA1 would be expected. However no peptide was detected, suggesting that the presence of LtnM1 restricts the peptide production. This phenomenon was also observed by Cotter et al. (28), who was unable to detect alanine-substituted peptides, such as LtnA1C29A, LtnA2C20A, LtnA2C25A and LtnA2C29A. It is unclear if the lack of peptide production is due to an deficiency in modification or blockage of exportation.

Lantibiotics are active only after the removal of the leader peptide. In class II Lantibiotics, processing involves a N-terminal peptidase domain that is present in the LanT transporter. When the lanthipeptide modifying system is introduced in a heterologous host for the production of unrelated and uncharacterized lantibiotics, it is not desirable that the putative lantibiotic is produced in an active form as this may kill the production strain. To overcome this problem with the class I lantibiotics production system based on the NisBC and NisT system, a protease site has been introduced into the leader peptide sequence that can be specifically cleaved by a protease using a post-production processing step. This was successful with the factor Xa processing site, which is a short and specific amino acid sequence recognized by the factor Xa protease (26). Using the same approach for the production of unprocessed LtnA1 failed. Either the introduction of the factor Xa protease site in between the leader and the core peptide or replacing the last four amino acids of the leader peptide by the specific IDGR sequence completely blocked the production of LtnA1. Unfortunately, in this experimental setup it is not possible to determine at which stage production was stopped. It could well be that there is intracellular accumulation of the dehydrated peptide because transport is blocked or modification is not taking place due to substrate specificity of LtnM1. The leader peptide of LtnA1 contains the double glycine motif (i.e., Gly-Ala) at its C-terminus. Site-directed mutagenesis studies showed that the presence of the double glycine motif is essential for the proteolytic removal of the leader peptide by LanT (29, 30), but such modifications did not interfere with the post-translational modifications of the core peptide (31). It seems that the LtnM1/LtnT system is highly specific and disruption of the double glycine motif or modification of the C-terminal region of the leader peptide, interferes with production and maturation and this severely limits the use of LtnM1/LtnT as a generic production system of class II lantibiotics.

Overall it can be concluded that the modification enzymes and the transporter are relatively specific, and that modifications in the substrate peptide readily result in a loss of production. Therefore, LtnM1, LtnM2 and LtnT are not suitable candidates to be used in bioengineering and the development of a class II lanthipeptide production system. Further insight is needed in the mechanism LanM enzymes employ to introduce (methyl)lanthionine residues and the

substrate recognition by these enzymes before they could be used for the bioengineering of lantibiotics.

Table 4: Schematic overview of detected peptides, by means of MALDI-TOF mass spectrometry, in supernatant of *L. lactis* NZ9000 co-expressing LtnT, LtnM1/LtnT or LtnM2/LtnT in combination with the indicated substrate variants.

	ltnT	**LtnM1/LtnT**	**LtnM2/LtnT**
Wild type peptides			
LtnA1	3424.59 Da *	3301.62 Da *	X
LtnA2	2982.85 Da *	X	2844.24 Da *
Fusion peptides			
LtnA1-A2	X	X	X
LtnA2-A1	X	X	X
Cysteine less peptides			
LtnA1(C-less)	3299.30 Da	X	N/A
LtnA2(C-less)	X	N/A	X
LtnA1-A2(C-less)	X	X	N/A
LtnA2-A1(C-less)	3298.99 Da	N/A	3293.36 Da
Factor Xa containing peptides			
LtnA1-xa1	X	X	N/A
LtnA1-xa2	X	X	N/A

1234.56 Da monoisotopic mass $(M+H)^+$ of detected the peptides

X no peptide detected by MALDI-TOF MS.

N/A not applicable/not determined

* results shown in chapter 2 and 3 of this thesis

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Chapter 5

Heterologous production of class II lantibiotics in *Escherichia coli*

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Abstract

Lantibiotics contain the unusual amino acids lanthionine or methyllanthionine, which are introduced post-translationally by lantibiotic synthetases. Many of these peptides act as antimicrobial agents. In class II lantibiotics the synthetase, LanM, is a bifunctional enzyme that performs both the dehydration of serine and threonine residues as well as the cyclization reaction that connects the dehydrated residue to a cysteine forming the thioether rings. To examine the potential of these modification enzymes in bioengineering of peptides, two such synthetase enzymes, MutM and LtnM2, were heterologously expressed in *E. coli* and their substrate specificity was explored. In the presence of MutM and LtnM2, fully dehydrated MutA and LtnA2 could be detected. When the native leader peptide of LtnA1, NisA, or LtnA2 was replaced by the leader peptide of MutA or LtnA2, MutM and LtnM2 supported various degrees of dehydration. These results suggest that the modification enzymes MutM and LtnM2 are capable of dehydrating a diverse range of peptides.

Introduction

Lanthipeptides are ribosomally synthesized and post-translationally modified peptides (RiPPs) that contain lanthionine or methyllanthionine residues. Lantibiotics, lanthionine-containing antibiotic peptides (1), are a subclass of lanthipeptides that exhibit antimicrobial activity. The production of lantibiotics is specified by gene clusters that can be found on plasmids, conjugative transposable elements or on the chromosome of the producing organism (2). The precursor peptide, encoded by *lanA*, can be divided in two parts, the N-terminal leader peptide and the C-terminal core peptide that is post-translationally modified. The biosynthetic gene cluster also contains genes encoding the modification enzymes, e.g. *lanB*, *lanC, lanM lanD and lanJ*, a transporter, *lanT*, a protease, *lanP*, proteins for immunity, e.g. *lanI*, *lanH*, *lanEFG*, and regulatory genes, *lanR*, *lanK*.

A lanthionine or methyllanthionine residue is formed by the dehydration of serine and threonine residues. The resulting dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively, are then coupled to a cysteine via a thioether bond. Based on the modification enzymes that introduce these modifications, lantibiotics can be divide in two classes (3, 4). In class I, including nisin as the paradigm, the dehydration is performed by the dehydratase LanB and the cyclization is catalyzed by a cyclase termed LanC. In class II lantibiotics, like mutacin II, there is a bifunctional modification enzyme, LanM, that performs both the dehydration and cyclization reaction. The cyclase domain of LanM shows homology to the LanC cyclase enzymes. However the N-terminal dehydratase domain does not show significant sequence identity with LanB (5).

Class I and II lantibiotics further differ in the mechanisms by which transport and processing of the precursor peptide occurs. In class I lantibiotics, the modified precursor peptides are transported across the membrane and thereafter processed by an extracellular protease (LanP) releasing the mature peptide. With class II lantibiotics, export and processing is done by a bifuntional transporter (LanT). The transporter contains an N-terminal intercellular C39 peptidase domain that removes the leader peptide concomitant with transport across the membrane. The leader peptides of class II lantibiotics contains a double glycine motif, GG, GA, GS or GT, at position -2 and -1 from the cleavage site, which is recognized by the C39 peptidase domain.

Lantibiotics exhibit antimicrobial activity mostly against Gram-positive bacteria. Gram-negative bacteria are usually not affected as the lantibiotics cannot permeate the outer membrane. Lantibiotics interfere with cell wall biosynthesis via binding to lipid II and/or disrupt the membrane integrity by pore formation. Since lipid II is the target, which is an essential intermediate in cell wall biosynthesis, there is only a low probability of generating resistance. In addition, (methyl)lanthionine residues are believed to increase the biostability and resistance against proteolytic degradation (6, 7), making them an interesting substance as antimicrobial agents.

The lantibiotic biosynthesis genes may be used for bioengineering in order to design new antimicrobial peptides or by providing existing peptides with a novel activity or biostability. Multiple studies showed that lantibiotic modification enzymes can be used for this purpose when expressed in *L. lactis*, e.g. NisB, NisC and NisT (8), LtnM2 and LtnT (9), LtnM1 and LtnT (Chapter 2) or nukacin ISK-1 (10). A heterologous expression system in *E. coli* could be an advantage over a production system such as *L. lactis* using the versatility of the genetic systems, the potential for high throughput applications as well as the insensitivity towards the expressing strain. Some examples of lanthipeptides that are heterologous expressed in *E. coli* are nukacin ISK-1 (11), nisin, prochlorosins and haloduracin (12). Here, mutacin II and the β-peptide of lacticin 3147 were co-expressed with their corresponding modification enzymes in *E. coli* to examine if these class II lantibiotics can be produced in this heterologous host. In addition, the substrate specificity of the modification enzymes, MutM and LtnM2 was studied.

Material and methods

Bacterial strains and growth conditions. Strains and plasmids used are listed in table 1. *Escherichia coli* DH5α was used for cloning and maintenance of plasmids. *E. coli* BL21 (DE3) was used as an expression host. Cells were cultured in Luria Broth (LB), and depending on the plasmid, supplemented with kanamycin (30 µg/ml) and/or ampicillin (50 µg/ml). *Streptococcus mutans* T8 (Kind gift from Department of Microbial Genomic, Université Laval, Québec QC Canada) was grown in trypton soya broth (Oxoid) at 37°C.

Table 1: List of strains and plasmids used in this study

	Characteristics	**reference**
Strains		
E. coli DH5α	Used for cloning and plasmid maintenance	
E. coli BL21 (DE3)	Used for protein expression	
Streptococcus mutans T8	Strain containing mutacin II gene cluster	Kind gift from the Department of Microbial Genomic, Université Laval, Québec QC Canada
Plasmids		
pETDuet-1	Amp^r; contains two multiple cloning sites (MCS) under control of T7*lac* promoter	Novagen
pETD-LtnM2a	Amp^r; pETDuet-1 derived; *ltnM2* cloned in MCS2	This study
pETD-MutM	Amp^r; pETDuet-1 derived; *mutM* cloned in MCS2	This study
pRSFDuet-1	Kan^r; contains two multiple cloning sites (MCS) under control of T7*lac* promoter	Novagen
pRSFD-ltnA2-M2a	Kan^r; pRSFDuet-1 derived; *ltnA2* cloned in MCS1, *ltnM2* cloned in MCS2	This study
pRSFD-LtnA2-NisA	Kan^r; pRSFDuet-1 derived; LtnA2(1-36)::NisA(24-57) cloned in MCS1	This study
pRSFD-mutA-M	Kan^r; pRSFDuet-1 derived; *mutA* cloned in MCS1, *mutM* cloned in MCS2	This study
pRSFD-mut-LtnA1-b	Kan^r; pRSFDuet-1 derived; mutA(1-26)::LtnA1(30-59) cloned in MCS1	This study
pRSFD-mut-nisA-b	Kan^r; pRSFDuet-1 derived; mutA(1-26)::NisA(24-57) cloned in MCS1	This study
pRSFD-mut-LtnA2	Kan^r; pRSFDuet-1 derived; mutA(1-26)::LtnA2(37-65) cloned in MCS1	This study

Kan^r: kanamycin resistance gene
Amp^r: ampicillin resistance gene

Construction of plasmids. Molecular cloning was performed using standard procedures (13). Phusion High-Fidelity DNA Polymerase for PCR, restriction and DNA modifying enzymes were all purchased from Thermo Scientific. Primer sequences for the PCR reactions are listed in table 2 and supplementary table 2.

To create pRSFD-mutA-M, genes encoding *mutA* and *mutM* from *S. mutans* T8 were amplified by PCR and cloned into the multiple cloning sites (MSC) 1 and 2 of pRSFDuet-1 (Novagen), respectively (for details see supplementary information). To create pETD-mutM the PCR product of *mutM* was

cloned into EcoRV and XhoI digested pETDuet-1 (Novagen) (see supplementary information).

To create pRSFD-mut-LtnA1-b and pRSFD-mut-NisA-b synthetic constructs containing MutA(-26 to -1)::LtnA1(1 to 30) or MutA(-26 to -1)::NisA(1 to 34) with a BamHI restriction site 5'-end and a HindIII restriction site 3'-end were ordered from GeneArt (Life Technologies). The synthetic DNA was used as template in a PCR, the PCR product was treated with BamHI and HindIII and placed in MCS 1 of pRSFDuet-1. Subsequently the resulting plasmids were subjected in mutagenesis PCR to place the fusion gene in frame behind the six histidine tag sequence. To create pRSFD-mut-ltnA2 a synthetic construct was ordered from GeneArt (Life Technologies) containing MutA(-26 to -1)::IDGR::LtnA2(1 to 29) with a BamHI restriction site 5'-end and a HindIII restriction site 3'-end of the fusion gene. The gene was amplified by PCR and cloned in MCS 1 of pRSFDuet-1. The resulting plasmid was used in mutagenesis PCR to remove the factor Xa protease site (IDGR). All the clones were analyzed by DNA sequence analysis at Macrogen Corporation.

Table 2: Primers used in this study

Primer	**Sequence (5'-3')**	**Characteristics**
19-fw	TAATACGACTCACTATAGGGC	Amplification synthetic constructs
20-rv	GACAGGTTTCCCGACTGGAAAG	Amplification synthetic constructs
151-mut-ltna1-fw	CAGCCAGGATCCGATGAATAAACTG	Placing fusion gene, *mut-ltnA1* and *mut-nisA* in frame behind His_6-tag
152-mut-ltna1-rv	CAGTTTATTCATCGGATCCTGGCTG	Placing fusion gene, *mut-ltnA1* and *mut-nisA* in frame behind His_6-tag
149-mut-ltna2-rv	ACCACCCAGAATGGTATCCAG	Phosphorylated 5'-end, primer to remove factor Xa-site
150-mut-ltna2-fw	ACCACTCCGGCAACACC	primer to remove factor Xa-site
063-pA24-fw	AGCTGGATCCGATGAAAGAAAAAAATATGAAAAAGAATG	introduction BamHI site 5'-end of *ltnA2*
064-pnz-3-rv	CTTATGGGATTTATCGAAAGCTTG	introduction HindIII site 3'-end of *ltnA2*
067-ltnm2-a-fw	AGTAGCATATGTTGGACCCATCAATAAAAAAATTAG	introduction NdeI site 5'-end of *ltnM2*
069-ltnm2-rv	ATTCTAGACTCGAGAGCTCTTAG	introduction XhoI site 3'-end of *ltnM2*
146-ltna2-muta-rv	ACCACCATGGCTTTCATCAC	Phosphorylated 5'-end, forward primer to remove factor Xa-site
148-ltna2-nisa-fw	ATTACCAGCATTAGCCTGTGTAC	Reverse primer to remove factor Xa-site

ATCG marking the sequence of the corresponding restriction site.

For the construction of pRSFD-LtnA2-M2, pA24 (chapter 2, kind gift from Lanthio Pharma (9)) harboring the gene *ltnA2* was used as a template to amplify *ltnA2* with a BamHI restriction site 5'-end and HindIII restriction site 3'-end. The PCR product was cloned in the MCS 1 of pRSFDuet-1. pILPTM2-2 (chapter 2, kind gift from Lanthio Pharma (9)) was used as template to amplify *ltnM2* with NdeI restriction site 5'-end and a XhoI restriction site 3'-end. Subsequently, the gene was cloned in the MSC 2 of pRSFD-LtnA2. In order to create pETD-ltnM2a the PCR product of *ltnM2* was ligated in MCS 2 of pETDuet-1.

To generate pRSFD-ltnA2-nisA, a synthetic construct was ordered from GeneArt (Life Technologies) containing LtnA2(-1 to -36)::IDGR::NisA(1 to 34) with a BamHI restriction site 5'-end and a HindIII restriction site 3'-end of the gene. The gene was amplified by PCR and cloned in MCS 1 of pRSFDuet-1. The resulting plasmid was then used in mutagenesis PCR to remove the factor Xa protease site (IDGR). The clones were analyzed by DNA sequence analysis at Macrogen Corporation.

Overexpression and purification of His-tagged lanthipeptides. *E. coli* BL21 (DE3) cells were transformed with pRSFDuet-1 or co-transformed with pRSFDuet-1 and pETDuet-1 derived plasmids containing a specific *lanA* and *lanM* gene (see table 1 and 3). Cells were plated on LB agar containing 50 µg/mL ampicillin and/or 30 µg/mL kanamycin depending on the plasmids used and overnight incubated at 37°C. Colonies of the resulting plate were resuspended in 1 mL LB, and used to inoculate 100 mL LB supplemented with appropriate antibiotics. Cells were grown at 37°C until the optical density at 600 nm (OD_{600}) was between 0.5 and 0.7. The culture was then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for two hours at 37°C (cells expressing LtnM2) or overnight at 20°C (cells expressing MutM). Cells were harvested by centrifugation at 2851 x g for 20 minutes (4°C), and stored as pellets at -20°C. Cell pellets were resuspended in 10 ml start buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) and lysed through sonication. Cell debris was removed by centrifugation (50 min. at 444,000 x g), and peptides were purified from the supernatant by immobilized metal affinity chromatography (IMAC) using a HisTrap™ HP column (GE Healthcare) with elution buffer (start buffer containing 250 mM imidazole). Fractions were analyzed by 15% Tris-tricine SDS-PAGE and

Coomassie brilliant blue staining, and by Western blotting using an anti-His antibody (5 PRIME) following standard protocols.

HPLC purification and mass spectrometry analysis. After IMAC-purification trifluor acidic acid (TFA) was added to the peptide containing fraction until pH 4 and further purified/desalted by reversed phase HPLC using a Jupiter Proteo C12 column (4 µm; 90 Å; 250 mm x 4.6 mm). The following solvents were used for RP-HPLC, solvent A (0.1% TFA in MilliQ water) and solvent B (0.1% TFA in acetonitrile). The HPLC purified peptide fractions were analyzed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. A sample of 1 µL of the HPLC fractions was spotted on the MALDI-TOF target and directly covered by 1 µL of matrix (5 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile in 0.1% TFA in MilliQ water). The spots were allowed to dry before spectra were recorded with a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). An external calibration was used to correct the resulting spectra.

Protease treatment of purified peptides. HPLC fractions containing the peptide were vacuum-dried using a Concentrator plus (Eppendorf). The pelleted samples, containing His_6-MutA or His_6-Mut-NisA, were resuspended in 500 µL 0.1 M Tris-HCl pH 8 containing 100 µg/mL trypsin (Sigma-Aldrich). The pelleted samples, containing His_6-LtnA2, His_6-Mut-LtnA1 or His_6-Mut-LtnA2, were resuspended in 500 µL 0.1 M ammonium acetate pH 4 containing 50 µg/mL endoproteinase Glu-C from *Staphylococcus aureus* V8 (Sigma-Aldrich). Samples were incubated three hours till overnight at 37°C. After protease treatment TFA (0.1%) was added and the peptide fragments were separated by HPLC and analyzed by MALDI-TOF MS as described above.

Results

Heterologous co-expression of *Streptococcus mutans* MutM and MutA in *E. coli*. To explore the use of the modification enzyme of mutacin II for the heterologous production of class II lantibiotics in *E. coli*, the genes of the modification enzyme, *mutM*, and its natural substrate, *mutA*, were cloned in

Table 3: Amino acid sequence and mass of proteins and chimera proteins heterologously co-expressed with MutM or LtnM2 in *E. coli*.

	His-tag sequence	Lantibiotic leader peptide	Lantibiotic core peptide	Mass (Da)*
Co-expressed with MutM:				
	1 5 10	15 20 25 30 35 40	45 50 55 60 65	
His$_6$-MutA	MGSSHHHHHHSQDP	MNKLNSNAVVSLNEVSDSELDTILGG	NRWWQGVVPTVSYECRMNSWQHVFTCC	7502.2449
	1 5 10	15 20 25 30 35 40	45 50 55 60 65 70	
His$_6$-Mut-LtnA1	MGSSHHHHHHSQDP	MNKLNSNAVVSLNEVSDSELDTILGG	CSINTFSLSDYWGNNGAWCTLTHECMAWCK	7615.2836
	1 5 10	15 20 25 30 35 40	45 50 55 60 65 70	
His$_6$-Mut-NisA	MGSSHHHHHHSQDP	MNKLNSNAVVSLNEVSDSELDTILGG	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	7682.6631
	1 5 10	15 20 25 30 35 40	45 50 55 60 65	
His$_6$-Mut-LtnA2	MGSSHHHHHHSQDP	MNKLNSNAVVSLNEVSDSELDTILGG	TTPATPAISILSAYISTNTCPTTKCTRAC	7171.9137
Co-expressed with LtnM2:				
	1 5 10	15 20 25 30 35 40 45 50	55 60 65 70 75	
His$_6$-LtnA2	MGSSHHHHHHSQDP	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG	TTPATPAISILSAYISTNTCPTTKCTRAC	8562.5054
	1 5 10	15 20 25 30 35 40 45 50	55 60 65 70 75 80	
His$_6$-LtnA2-NisA	MGSSHHHHHHSQDP	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	9073.2547

* The average mass of the proteins, $(M+H)^+$, was calculated without the initial methionine.
TS Ser or Thr residues that are dehydrated in wild type situation

a pRSFDuet-1 vector. MutA was fused at its N-terminus to a six histidine-tag encoding sequence creating His_6-MutA. After co-expression in *E. coli* BL21 (DE3), cells of the expressing strain were analyzed for the presence of His_6-MutA. Herein, the cell free extract was passed over an immobilized metal affinity chromatography (IMAC) column and analyzed by Tris-tricine SDS-PAGE and Coomassie brilliant blue staining. This showed the presence of a protein (~10 kDa) that was absent in the empty vector control (figure 1A). The protein was stained by Western blotting using a Tetra-His antibody (5 prime), confirming the presence of His-tagged MutA (figure 1B).

The IMAC fraction that contained the peptide was applied on RP-HPLC for further purification. HPLC fractions were collected and analyzed by MALDI-TOF mass spectrometry for the presence of His_6-MutA. The HPLC fraction around 24 min. retention time contained a peak of 7421.13 Da (figure 2A/table 4). This mass may indicate the presence of a four times dehydrated His_6-MutA with a theoretical mass of 7430.1649 Da. Since the observed and calculated mass differ around 9 Da, the fraction containing the peptide was digested with trypsin, herewith confirming the presence of His_6-MutA and to get more insight in the precise number of dehydrations. After digestion and RP-HPLC, fractions were analyzed by MALDI-TOF mass spectrometry. One of the HPLC fractions contained peaks that could be assigned to amino acids Leu18-Arg42 and Trp43-Cys67 from His_6-MutA (figure 3A/table 4). The core peptide of mutacin II, corresponding to Asn41-Cys67 in His_6-MutA (table 3), contains four Ser and Thr residues that are all dehydrated. The calculated mass of the peptide fragment Trp43-Cys67 is 3046.3261 Da, and a peak corresponding to the four times dehydrated fragment was present (figure 3A/table 4). In addition to the fully dehydrated peptide, also incomplete dehydration was observed ranging from none to three times dehydration. The results suggest that MutM is expressed and active in *E. coli* yielding dehydrated MutA.

MutA chimeric peptides are expressed and dehydrated in *E. coli*. In order to validate if MutM is also capable to dehydrate unrelated peptides a set of chimeric peptides was generated (table 3). The chimeric peptides consist of the leader peptide of MutA fused to the core peptides of LtnA1, LtnA2 or NisA. The respective genes were co-expressed as his-tagged chimeric peptides together

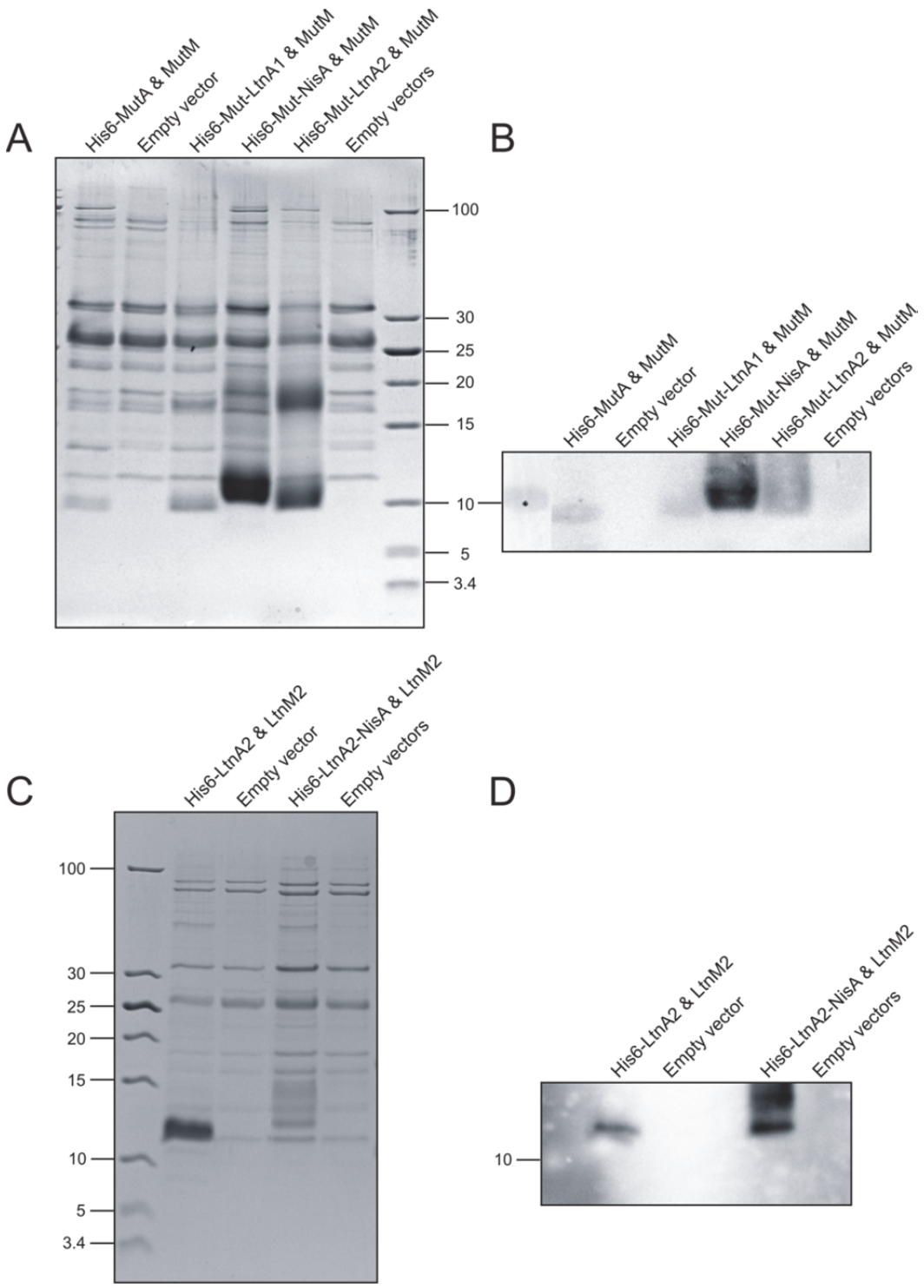

Figure 1: Expression of LanM enzymes and production of LanA products in *E. coli*. Tris-tricine Coomassie stained SDS-PAGE comparing elution fractions after IMAC purification of *E. coli* strains expressing His_6-MutA and MutM, His_6-Mut-LtnA1 and MutM, His_6-Mut-NisA and MutM, His_6-Mut-LtnA2 and MutM or empty vectors (A). Western blot stained with a Tetra-His antibody (Qiagen) showing the presence of his-tagged proteins (~9 and ~10 kDa) in the elution fractions after IMAC purification (B). Tris-tricine Coomassie stained SDS-PAGE comparing elution fractions after IMAC purification of *E. coli* strains expressing His_6-LtnA2 and LtnM2, His_6-LtnA2-NisA and LtnM2 or empty vectors (C). Western blot stained with a Tetra-His antibody (Qiagen) showing the presence of his-tagged proteins (~12 kDa) in the elution fractions after IMAC purification (D).

with the modification enzyme, MutM. After co-expression in *E. coli* BL21 (DE3), cell free extract was passed over an IMAC column to isolate the His-tagged peptides and analyzed by Tris-tricine SDS-PAGE and Coomassie staining. The elution fractions contained an extra protein (~10 kDa) compared to the empty vector control (figure 1A), that stained with an Tetra-His antibody (figure 1B). These data indicate the production of His_6-mut-LtnA1, His_6-mut-LtnA2 and His_6-mut-NisA. The peptide containing fractions were further purified over RP-HPLC, and subjected to MALDI-TOF mass spectrometry. This yielded unique masses of 7573.67 Da and 7545.19 Da for His_6-Mut-LtnA1 (figure 2B/table 4) that likely correspond to two and four times dehydrated His_6-Mut-LtnA1 which has a theoretical mass of 7615.2836 Da when unmodified. The theoretical mass of unmodified His_6-Mut-NisA is 7682.6631 Da, and mass analysis revealed a peak at 7607.31 Da which likely corresponds to four times dehydrated peptide (figure 2C/table 4). With His_6-Mut-ltnA2 a peak at 7129.20 Da (figure 2D/table 4) was found that likely corresponds to a two-fold dehydrated peptide that unmodified has a predicted mass of 7171.9137 Da.

To determine the degree of dehydration more accurate, the peptides were digested by Glu-C or trypsin and analyzed by RP-HPLC and MALDI-TOF mass spectrometry. Digestion of His_6-Mut-LtnA1 with Glu-C yielded peaks corresponding to two, three and four times dehydrated peptide fragment Leu34-Lys70 (figure 3C/table 4). His_6-Mut-NisA was digested with trypsin, yielding a NisA fragment (Thr53-Lys74) that was dehydrated once (figure 3D/table 4). His_6-Mut-LtnA2 digested with Glu-C yielded the fragment of Leu34-Gly69 with the two masses of 3530.90 Da and 3511.80 Da corresponding to seven and eight times dehydrated peptide (figure 3E/table 4). These results suggest that MutM is able to modify unrelated peptide substrates when expressed as a chimeric peptide with the mutacin II leader peptide.

Heterologous co-expression of lacticin 3147 LtnM2 and its natural substrate in *E. coli*. In addition to MutM, the modification enzyme LtnM2 of the two-component class II lantibiotic lacticin 3147, was explored for heterologous expression in *E. coli*. The genes encoding the modification enzyme, LtnM2, and a hexa-his tag variant of the natural substrate, LtnA2, were cloned and expressed in *E. coli* BL21 (DE3). Analysis of the cell free extract after IMAC column chromatography yielded a unique 12 kDa protein band on Coomassie stained

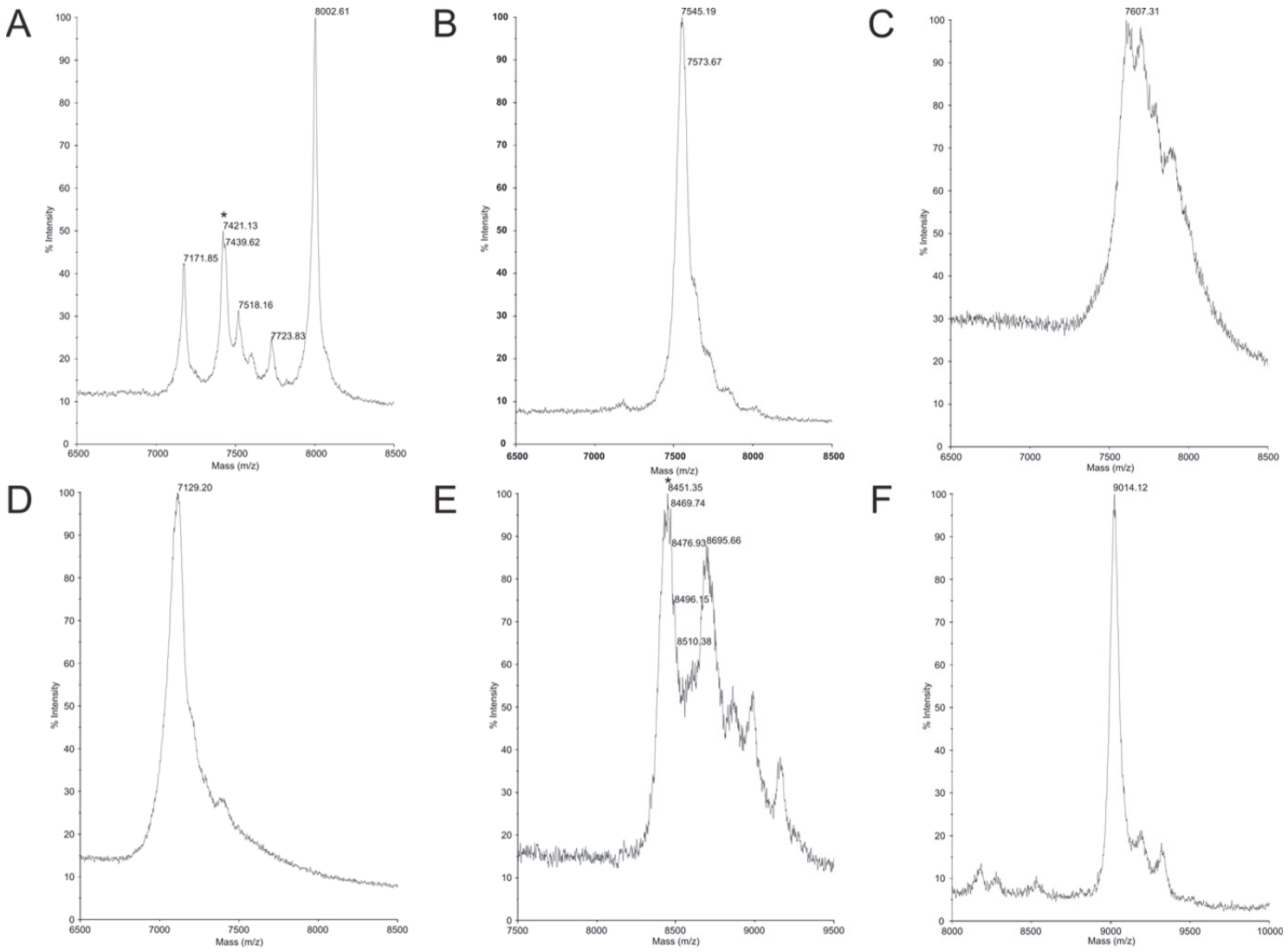

Figure 2: MALDI-TOF mass spectra of HPLC fractions containing peptides after co-expression of MutM with His_6-MutA (A), His_6-Mut-LtnA1 (B), His_6-Mut-NisA (C) and His_6-Mut-LtnA2 (D), and after co-expression of LtnM2 with His_6-LtnA2 (E) and His_6-LtnA2-NisA (F).

Tris-tricine SDS-PAGE that is absent in the empty vector control (figure 1C). This band was also stained with a Tetra-His antibody, confirming the presence of His-tagged LtnA2 (figure 1D). The peptide was purified using a IMAC column and RP-HPLC, thereafter analyzed by MALDI-TOF mass spectrometry for the presence of His_6-LtnA2. One of the fractions contained a peak at 8451.35 Da (figure 2E/table 4) corresponding to one time dehydrated His_6-LtnA2 with a theoretical mass of 8562.5054 Da when unmodified. The fraction was digested with Endoproteinase Glu-C to reveal more exact the number of dehydrations resulting in the following peptide fractions: Leu41-Cys79, Gly44-Cys79 and Ser47-Cys79. The calculated masses of these peptides are 3938.8574, 3625.6936 and 3324.6026 Da, respectively. The observed masses 3822.36, 3479.01 and 3179.87 Da (figure 3B/table 4) likely correspond to peptide fragments that are eight times dehydrated which is identical to the number of dehydrations found in the native LtnA2. In addition peptide fragments with incomplete dehydration, ranging from none to seven times, were observed (data not shown). Finding dehydrated His_6-LtnA2 proposes an active LtnM2 when expressed in *E. coli*.

Expression of His_6-LtnA2-NisA chimera peptide in *E. coli*. To investigate the ability of LtnM2 to modify peptides other than its native substrate LtnA2, His_6-LtnA2-NisA was tested. Again, gel-based analysis revealed a unique protein band around 12 kDa, absent in the empty vector control sample (figure 1C), and immunoreactive with a Tetra-His antibody (figure 1D). RP-HPLC and MALDI-TOF mass spectrometry revealed a peak at 9014.12 Da (figure 2F/table 4) in one of the fractions that correspond to three times dehydrated His_6-LtnA2-NisA (theoretical mass 9073.2547 Da). After Glu-C proteinase digestion, no peptide fragment could be recovered, likely because the lower levels of expression of the protein. These data however show production and dehydration of His_6-LtnA2-NisA when co-expressed with LtnM2 in *E. coli*.

Discussion

Bacteria produce a large variety of lanthipeptides in nature, peptides that vary in amino acid sequence, degree of dehydration and number of thioether bonds. Although the basic enzymatic machinery is shared among these lanthipeptides, the exact specificity of the modifying enzymes is unknown.

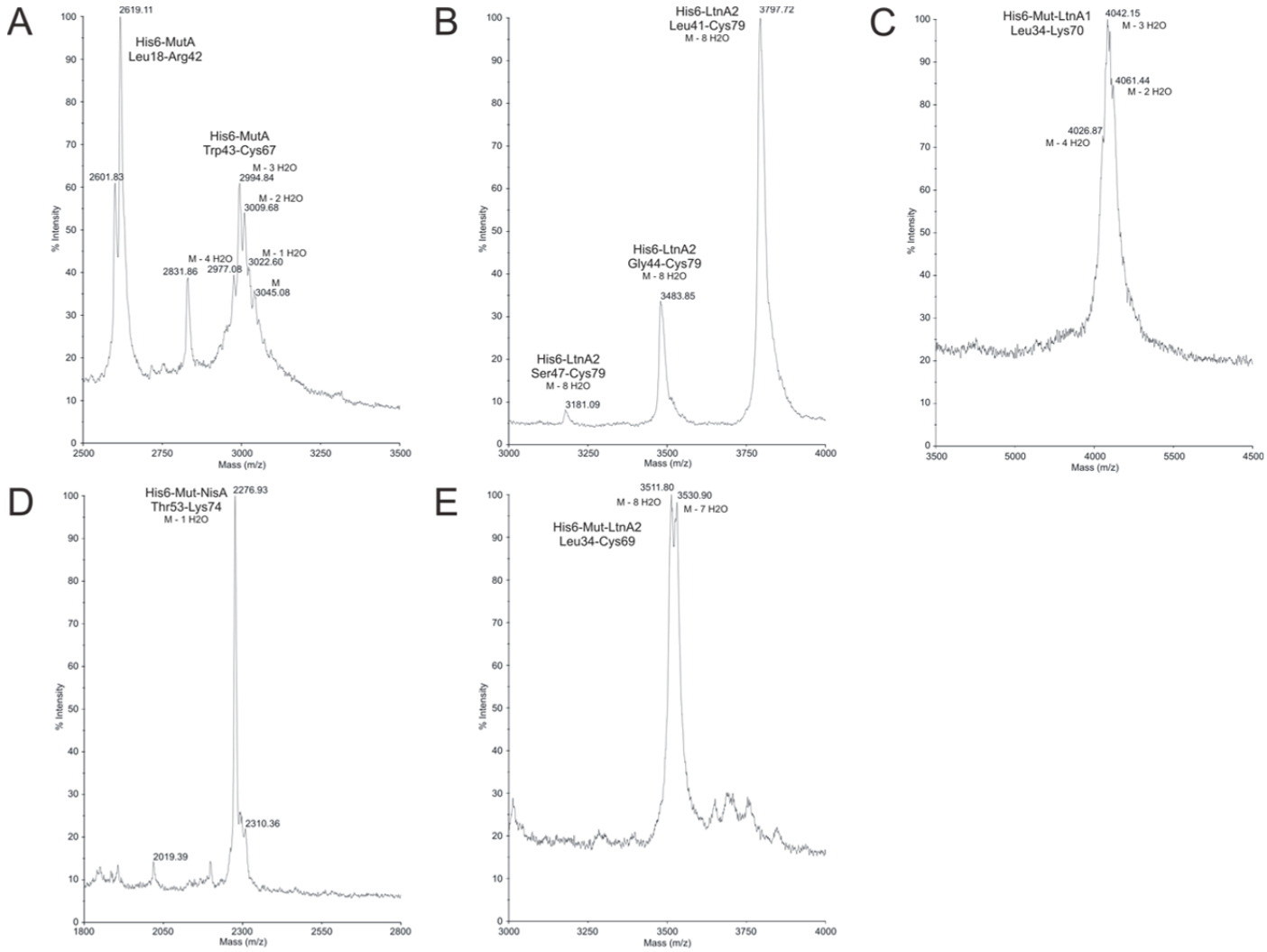

Figure 3: MALDI-TOF mass spectra of (endo)proteinase digested peptides showing the peaks corresponding to peptide fragments: Leu18-Arg42 and Trp43-Cys67 of dehydrated His_6-MutA (A), Ser47-Cys79, Gly44-Cys79 and Leu41-Cys79 of dehydrated His_6-LtnA2 (B), Leu34-Lys70 of dehydrated His_6-Mut-LtnA1 (C), Thr53-Lys74 of dehydrated His_6-Mut-NisA (D) or Leu34-Cys69 of dehydrated His_6-Mut-LtnA2 (E).

Several successes have been reported for class I lantibiotics where the NisBC enzymes were used to modify unrelated lanthipeptides as well as small peptide hormones (14, 15). In addition, one example has been described suggesting that LtnM2, from the class II lantibiotic lacticin 3147, has a tolerance for unrelated lanthipeptides (9). However, there are no further reports of a production system that depends on the single modifying enzyme, LanM, carrying out both dehydration and thioether-link formation. Little is known about the promiscuity of these LanM enzymes. On the other hand, such modifying enzymes constitute an engineering potential, possibly allowing the production of a vast range of lanthipeptides when co-expressed with structural LanA-like peptides. To explore this possibility, MutM and LtnM2 were co-expressed with several LanA peptides in *E. coli*, and analyzed on the production of peptides by HPLC and MALDI-TOF mass spectrometry. This study could show that MutM fully dehydrates (4-times) its cognate substrate MutA, that was co-expressed as a His-tagged protein. Similarly, LtnM2 was shown to fully dehydrate (8-times) LtnA2. This shows that the modification enzymes are active when expressed in a heterologous host like *E. coli*. In addition LtnM2 and MutM seem to be tolerant to the presence of a N-terminal His-tag. This was shown before for NukM (11), ProcM, the haloduracin and nisin modification enzymes (12). Moreover, the leader peptide of MutA and LtnA2 was fused to a set of alternative LanA-like peptides to test the substrate promiscuity of LtnM2 and MutM, respectively. Indeed, MutM was shown to dehydrate His_6-Mut-NisA once, His_6-Mut-LtnA1 four times, and His_6-Mut-LtnA2 eight times. On the other hand, His_6-LtnA2-NisA was dehydrated three times by LtnM2.

Our work focuses on the development of an expression system for class II lanthipeptides. The intended production system should contain a known and promiscuous lantibiotic modification enzyme. For LtnM2, both expression in the Gram-positive host *L. lactis* and Gram-negative host *E. coli* as modifying enzyme was evaluated. By the co-expression of LtnM2, the transporter LtnT and various substrates in *L. lactis,* some peptides (LtnA2 and LtnA2-A1(C-less)) could be produced, but most attempts failed (Chapter 4). This suggest that LtnM2 in conjunction with LtnT is not suitable as a bioengineering enzymes for a class II lanthipeptide production system. This study, however, suggests that the production loss could be due to the substrate specificity of the transporter and might not be solely contributed to LtnM2. Although, different substrate variants

could be co-expressed with LtnM2, the enzymes does not seem highly specific. From the results described here it can be concluded that MutM and LtnM2 are capable of dehydrating unrelated LanA peptides when fused behind an appropriate leader peptide. However, the extent of dehydration varies from the expected number to under dehydration suggesting that further tuning is necessary to develop *E. coli* into a generic production host for class II lanthipeptides.

Table 4: Overview of the observed and theoretical masses of the full peptides and peptide fragments found during MALDI-TOF mass spectrometry analysis displayed in figure 2 and 3.

Full peptide/ peptide fragment	Observed Mass (Da)	Theoretical mass (Da)	Δ mass (Da)
His_6-MutA + MutM			
His_6-MutA	7421.13	7430.16 (4x)*	-9.03
Leu18-Arg42	2619.11	2616.31 **	+2.80
Trp43-Cys67	3045.08	3046.32 (0x)**	-1.24
	3022.60	3028.30 (1x)	-5.70
	3009.68	3010.28 (2x)	-0.60
	2994.84	2992.26 (3x)	+2.58
	2977.08	2974.24 (4x)	+2.84
His_6-Mut-LtnA1 + MutM			
His_6-Mut-LtnA1	7573.67	7579.24 (2x)*	-5.57
	7545.19	7543.20 (4x)	+1.99
Leu34-Lys70	4061.44	4062.71 (2x)*	-1.27
	4042.15	4044.69 (3x)	-2.54
	4026.87	4026.67 (4x)	+0.20
His_6-Mut-NisA + MutM			
His_6-Mut-NisA	7607.31	7610.58 (4x)*	-3.27
Thr53-Lys74	2276.93	2275.01 (1x)*	+1.92
His_6-Mut-ltnA2 + MutM			
His_6-Mut-ltnA2	7129.20	7135.87 (2x)*	-6.67
Leu34-Gly69	3530.90	3529.69 (7x)*	+1.21
	3511.80	3511.67 (8x)	+0.13
His_6-LtnA2 + LtnM2			
His_6-LtnA2	8451.35	8454.38 (6x)*	-3.03
Leu41-Cys79	3797.72	3794.69 (8x)**	+3.03
Gly44-Cys79	3483.85	3481.53 (8x)**	+2.32
Ser47-Cys79	3181.09	3180.44 (8x)**	+0.65
His_6-LtnA2-NisA + LtnM2			
His_6-LtnA2-NisA	9014.12	9019.19 (3x)*	-5.07

* The average mass of the protein, $(M+H)^+$, calculated without the initial methionine.
** The monoisotopic mass of the peptides $(M+H)^+$.
1x Mass calculated after given amount of dehydrations have been subtracted. One dehydration reduces the mass with 18.02 Da.
Δ The difference in theoretical and observed mass.

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Supplementary information

Construction of plasmid pRSFD-mutA-M. Strains and plasmids used to create pRSFD-mutA-M are listed in supplementary table 1. *L. lactis* NZ9000 was used as a host for the pNZ8048 and pIL253 derived plasmids. Strains were grown at 30°C in Bacto M17 broth (Becton Dickinson Difco) supplemented with 0.5% glucose. Where needed chloramphenicol (5 µg/mL) or erythromycin (5 µg/mL) were added to the medium. Primer sequences for the PCR reactions described below are listed in supplementary table 2.

For the construction of pRSFD-mutA, the gene encoding *mutA* was amplified via colony PCR from *S. mutans* T8. The resulting PCR product was treated with PagI and PstI and ligated in pNZ8048 to yield pNZ-mutA-1. This plasmid was used in a follow up PCR reaction amplifying *mutA* with BamHI and HindIII restriction sites. The resulting PCR product was ligated in the multiple cloning site (MSC) 1 of pRSFDuet-1 (Novagen).

To construct pILP-mutM-1, the nisin promotor region was amplified via PCR from pNZ8048. The PCR product was treated with BamHI and EcoRI and ligated in pIL253, resulting in pIL-pnis. Next, the *mutM* encoding gene was amplified via PCR as described for *mutA*, treated with BamHI and SacI and ligated in pIL-pnis creating pILP-mutM-1.

For pRSFD-mutA-M, *mutM* was amplified by PCR from pILP-mutM-1 with a XhoI restriction site at the 3'-end and a blunt 5'-end, and ligated in MSC 2 of pRFSD-mutA treated with EcoRV and XhoI.

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Supplementary table 1: list of strains and plasmids used in creation of pRSFD-mutA-M

	Characteristics	**reference**
Strains		
Escherichia coli DH5α	Used for cloning and plasmid maintenance	
Lactococcus lactis NZ9000	MG1363 derivative; pepN::nisRK+ Used for cloning and plasmid maintenance	(1)
Streptococcus mutans T8	Strain containing mutacin II gene cluster	Kind gift from the Department of Microbial Genomic, Université Laval, Québec QC Canada
Plasmids		
pNZ8048	Cm^r	(1)
pNZ-mutA-1	Cm^r; pNZ8048 derived; *mutA* cloned behind the *Pnis* promoter	This study
pRSFDuet-1	Kan^r; contains two multiple cloning sites (MCS) under control of T7lac promoter	Novagen
pIL253	Em^r	(2)
pIL-pnis	Em^r; pIL253 derived; containing *Pnis* promoter of pNZ8048	This study
pILP-mutM	Em^r; pIL253 derived; containing *mutM* behind *Pnis* promoter of pNZ8048	This study
pRSFD-mutA	Kan^r; pRSFDuet-1 derived; mutA cloned in MCS1, mutM cloned in MCS2	This study
pRSFD-mutA-M	Kan^r; pRSFDuet-1 derived; mutA cloned in MCS1, mutM cloned in MCS2	This study

Cm^r chloramphenicol resistance marker
Em^r erythromycin resistance marker
Kan^r: kanamycin resistance gene

Supplementary table 2: Primers used in creation of pRSFD-mutA-M

Primer	**Sequence (5'-3')**	**Characteristics**
050-mut-a1-fw	TAGCTCATGAACAAGTTAAACAGTAACG	Introduction PagI site 5'-end of *mutA*
051-mut-a2-rv	TAGCCTGCAGTTAACAGCAAGTGAAAACATG	Introduction PstI site 3'-end of *mutA*
086-muta-fw	AGTGAGGATCCCATGAACAAGTTAAACAGTAAC	Introduction BamHI site 5'-end of *mutA*
087-muta-rv	AGTACAAGCTTAACAGCAAGTGAAAACATG	Introduction HindIII site 3'-end of *mutA*
027-pnis-2-fw	CTTAGGGAATTCTAGTCTTATAACTATACTGAC	Introduction EcoRI site 5'-end of *Pnis* promoter
028-pnis-2-rv	CTTACTGGATCCGTGAGTGCCTCCTTATAATTTA	Introduction BamHI site 3'-end of *Pnis* promoter
081-mutm-fw	ATTAGGATCCATGAACAACCCGTTATTCCCTG	Introduction BamHI site 5'-end of *mutM*
082-mutm-rv	CTACGAGCTCTTAGTAAAATGAAAGATTTAAGACATG	Introduction of SacI site 3'-end of *mutM*
088-mutm-fw	CATGAACAACCCGTTATTCC	
089-mutm-rv	CTAGACTCGAGAGCTCTTAG	Introduction of XhoI site 3'-end of *mutM*

ATCG marking the sequence of the corresponding restriction site

Chapter 6

Expression and modification of the class II lantibiotic LanA-SPN23F in *Escherichia coli*

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Abstract

Lantibiotics are post-translationally modified peptides that function as antimicrobial agents. With class II lantibiotics, the bifunctional enzyme LanM both catalyzes the dehydration of serine and threonine, as well as the cyclization reaction that forms the thioether link between these residues and a cysteine. This study explored the ability of a putative LanM enzyme to modify its predicted class II lantibiotic substrate from *Streptococcus pneumoniae* Spain 23F-1 by heterologous expression in *Escherichia coli*. The his-tagged LanA-SPN23F protein was co-expressed with LanM-SPN23F in *E. coli*, and the resulting His_6-LanA-SPN23F was purified and examined by MALDI-TOF mass spectrometry for the presence of dehydrated residues. The peptide was found to be completely dehydrated, i.e. 7-8 times. Since the LanA-SPN23F peptide contains the Ile-Cys-Cys consensus sequence for a carboxyl-terminal decarboxylation modification, it was co-expressed with GdmD, a decarboxylation enzyme of *Staphylococcus gallinarum*, showing the decarboxylation in addition to dehydration.

Introduction

Lanthipeptides are ribosomally synthesized peptides that undergo post-translational modifications. They are characterized by the presence of the unusual amino acids lanthionine (Lan) and methyllanthionine (MeLan) (1, 2). Lanthipeptides can be divided into four classes based on the enzyme(s) that catalyze the formation of the lanthionine and methyllanthionine residues (1). Class I and II lanthipeptides are also termed lantibiotics, lanthionine-containing antibiotic peptides (3), since they possess antimicrobial activity.

Lanthipeptides are encoded by a gene generically termed, *lanA*. LanA contains a N-terminal leader and the C-terminal core peptide. The C-terminal core peptide is modified by distinct modification enzymes to introduce (methyl)lanthionine residues. Serine and threonine residues are dehydrated to form dehydroalanine (Dha) and dehydrobutrine (Dhb) residues, respectively. Subsequently, the dehydrated residues are coupled to a cysteine via a thioether bridge to form a lanthionine or a methyllanthionine, respectively. In class I lantibiotics the formation of these reactions are performed by two distinct enzymes, a dehydratase, LanB, and a cyclase, LanC. In class II lantibiotics the reaction is performed by a single bifunctional enzyme, LanM.

Besides the characteristic (methyl)lanthionine formation a lantibiotic can undergo more post-translational modifications (4). One of these extra modifications that can occur is the formation of an S-aminovinyl-D-cysteine (AviCys) amino acid. The enzyme responsible for the oxidative decarboxylation at the C-terminus of the lantibiotic is generically termed LanD. A more extensively studied LanD is EpiD from *Staphylococcus epidermidis*. EpiD belongs to the homooligomeric flavin-containing Cys decarboxylase (HFCD) family (5). The consensus sequence of the C-terminal amino acids for the recognition by EpiD is [V/I/L/F/W/Y/(M)]-[A/S/V/T/C/(I/L)]-C (6). Another example of a LanD enzyme is GdmD, which belongs to the gallidermin biosynthetic gene cluster of *Staphylococcus gallinarum*. Recently, this enzyme was shown to modify another lanthipeptide than gallidermin, namely a nisin variant, when co-expressed in *Lactococcus lactis* (7).

LanA peptides contain an N-terminal leader peptide. The precise function of this leader peptide is unknown (8), but it appears to play a significant role in targeting the peptide to the modification enzymes, export and self-immunity

systems. Lantibiotics belonging to class II contain the so-called double glycine motif (GG, GA, GS or GT) at the C-terminus of their leader peptide. This motif is recognized by the bifunctional transporter, LanT, typical for class II lantibiotics (9). This transporter contains a N-terminal peptidase C39 domain that is responsible for the cleavage of the leader peptide at the cytoplasmic side of the membrane whereupon the peptide is exported (9). It was previously shown that NukT requires ATP hydrolysis for leader cleavage activity to occur in vivo (10), though in vitro, the C39 peptidase domain of LctT can function as an independent protease (11).

Since multidrug resistance is a major problem in the treatment of infectious diseases, there is an urgent need for new antimicrobial substances based on unique structures. Lantibiotics possess antimicrobial activity and resistance against lantibiotics has not yet been reported, making them potential alterative candidates for a novel class of antibiotics (12). Thus far, lantibiotics are mainly used in food preservation preventing the growth of food pathogens. Due to genome-mining and bioinformatics, a multitude of lanthipeptide and lantibiotic associated gene clusters have been identified in the DNA databases. The bioactivities of these putative lantibiotics are not known. Often the genes are identified in metagenomic data and/or bacteria that so far have not been cultured. Therefore, expressing such new lantibiotics in their natural host is not feasible and ideally production should be realized using an heterologous host. Previously, it has been reported that the lantibiotic enzymes NukM (13), ProcM, HalM1, HalM2 and NisBC (14), and their substrates can be heterologously expressed in *Escherichia coli* to produce lanthipeptides. Therefore this study focuses on the heterologous expression of a putative class II lantibiotic from *Streptococcus pneumoniae* in *E. coli*. In addition, the substrate was subjected to an extra modification that entails a C-terminal decarboxylation.

Material and methods

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in table 1. *Escherichia coli* DH5α was used for cloning and maintenance of the plasmids, *E. coli* BL21 (DE3) was used as an expression host and Luria Broth (LB) was used as culture medium. Depending on the containing

plasmid, the LB was supplemented with kanamycin (30 µg/ml) and/or ampicillin (50 µg/ml).

Table 1: Strains and plasmids used in this study

	Characteristics	reference
Strains		
E. coli DH5α		
E. coli BL21 (DE3)		
Plasmids		
pETDuet-1	Amp^r; contains two multiple cloning sites (MCS) under control of T7*lac* promoter	Novagen
pETD-lanM	Amp^r; pETDuet-1 derived; *lanM* cloned in MCS2	This study
pETD-gdmD-lanM	Amp^r; pETDuet-1 derived; *gdmD* cloned in MCS1 and *lanM* cloned in MCS2	This study
pRSFDuet-1	Kan^r; contains two multiple cloning sites (MCS) under control of T7*lac* promoter	Novagen
pRSFD-LanA	Kan^r; pRSFDuet-1 derived; *lanA*-SPN23F cloned in MCS1	This study
pRSFD-lanTc39	Kan^r; pRSFDuet-1 derived; Leu1-Lys151 of LanT-SPN23F cloned in MCS1	This study
pNZE-gdmD	Ery^r; *gdmD* cloned behind the *Pnis* promoter	(7)

Kan^r: kanamycin resistance gene
Amp^r: ampicillin resistance gene

Construction of the plasmids. Chromosomal DNA isolated from *Streptococcus pneumoniae* Spain 23F-1 (ATCC 700669) was a kind gift from the Molecular Genetics department of the University of Groningen, and was used as a template to amplify the genes encoding *lanA-SPN23F*, *lanM-SPN23F* and the protease domain of *lanT-SPN23F*, LanTc39, using the primers listed in table 2. Phusion High-Fidelity DNA Polymerase for PCR, restriction and DNA modifying enzymes were all purchased from Thermo Scientific.

For the construction of pRSFD-LanA and pRSFD-LanTc39 the primers were designed such that a BamHI restriction site was added at the 5'-end and a HindIII restriction site at the 3'-end of the DNA. The *lanA-SPN23F* encoding gene and the protease domain of LanT-SPN23F were ligated in multiple cloning site 1 (MCS 1) of pRSFDuet-1 (Novagen), which was treated with the corresponding restriction enzymes. Since MCS 1 contains a His-tag, the cloning resulted in the addition of a N-terminal six histidine tag in front of the leader peptide of LanA-

SPN23F and LanTc39. Positive clones containing pRSFD-lanA or pRSFD-lanTc39, were analyzed by DNA sequence analysis at Macrogen Corporation.

For the construction of pETD-LanM the PCR primers for the *lanM-SPN23F* encoding gene introduce a BglII restriction site at the 5'-end of *lanM*, while at the 3'-end a natural occurring XhoI restriction site could be used. *LanM-SPN23F* was ligated in multiple cloning site 2 (MCS 2) of pETDuet-1 (Novagen), which was treated with corresponding restriction enzymes. pNZE-gdmD (7) was used as a template to amplify the gene encoding GdmD using primers that added a NcoI restriction site at the 5'-end and a HinDIII restriction site at the 3'-end of the gene (table 2). The fragment was cloned in MCS 1 of pETD-lanM (excluding the his-tag) to yield pETD-gdmD-lanM.

Table 2: Primers sequences used in this study

Primer	**Sequence (5'-3')**	**Characteristics**
090-lana-fw	AGTGAGGATCCCATGTCTGATTGGAAAAAAC	Introduction BamHI site 5'-end of *lanA-SPN23F*
091-lana-rv	AAAGAAAGCTTAACAACAAATGAATG	Introduction HindIII site 3'-end of *lanA-SPN23F*
135-lanTc39-fw	CAGTAGGGATCCATTGAAAAAGGTAAAATTCATACAAC	Introduction BamHI site 5'-end of *lanTc39*
136-lanTc39-rv	CTGATCAAGCTTACTTCAATATTTTAAAAAAATTACTATGC	Introduction HindIII site 3'-end of *lanTc39*
092-lanm-fw	ATGACAGATCTCATGAATCGAAAATTAAACATAAC	Introduction BglII site 5'-end of *lanM-SPN23F*
093-lanm-rv	AAATCGTATCGATTCTAGACTC	Introduction XhoI site 3'-end of *lanM-SPN23F*
124-gdmd-fw	AGTGACCATGGCGATGCATGGTAAATTATTGATATG	Introduction NcoI site 5'-end of *gdmD*
125-gdmd-rv	GATTCAAGCTTAGTCCAAAGGTCTCTTTTCG	Introduction HindIII site 3'-end of *gdmD*

ATCG marking the sequence of the corresponding restriction site.

Overexpression and purification of His_6-LanA-SPN23F. *E. coli* BL21 (DE3) cells were co-transformed with pRSFD-lanA and one of the following vectors; pETD-lanM or pETD-gdmD-lanM. After transformation, cells were plated on LB agar containing 30 µg/mL kanamycin and 50 µg/mL ampicillin. Colonies were resuspended in 1 mL LB and used to inoculate 100 mL LB supplemented with kanamycin and ampicillin. Cells were grown at 37°C until the optical density at 600 nm (OD_{600}) was between 0.5 and 0.7. The culture was then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for two hours at 37°C. Cells were harvested by centrifugation at 2851 x g for 20 minutes (4°C)

and stored as pellets at -20°C. Cell pellets were resuspended in 10 ml start buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) and lysed through sonication. Cell debris was removed by centrifugation (50 min. at 444,000 x g), and the peptides were purified from the supernatant by immobilized metal affinity chromatography (IMAC) using a HisTrap™ HP column (GE Healthcare) with elution buffer (start buffer containing 250 mM imidazole). Fractions were analyzed by 15% Tris-tricine SDS-PAGE and Coomassie brilliant blue staining and by Western blotting using an anti-His antibody (5 PRIME) following standard protocols.

HPLC purification and mass spectrometry analysis of His_6-LanA-SPN23F containing samples. After IMAC-purification trifluor acidic acid (TFA) was added to the peptide containing fraction until pH 4, whereupon the samples were purified and desalted by reversed phase HPLC using a Jupiter Proteo C12 column (4 µm; 90 Å; 250 mm x 4.6 mm). The following solvents were used for RP-HPLC, solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The HPLC purified peptide fractions were analyzed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on the presence of the peptide. A sample of 1 µL of the HPLC fractions was spotted on the target and directly covered by 1 µL of matrix (5 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile in 0.1% TFA in MQ). The spots were allowed to dry before spectra were recorded with a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). An external calibration was used to correct the resulting spectra.

Protease treatment of purified peptides. HPLC fractions containing the peptide were vacuum-dried using the Concentrator plus (Eppendorf). Pelleted samples were resuspended in 100 µL 0.5 M Tris-HCl pH 8 containing 0.5 mg/mL trypsin. Samples were incubated overnight at 37°C. After protease treatment TFA was added and the peptide fragments were separated by HPLC and analyzed by MALDI-TOF MS as described above.

Overexpression and purification of His_6-LanTc39. Overexpression of His_6-LanTc39 was essentially as described for His_6-LanA-SPN23F. *E. coli* BL21 (DE3) cells were transformed with pRSFD-lanTc39. After transformation the cells

were plated on LB agar containing 30 µg/mL kanamycin. Colonies were resuspended in 1 mL LB and used to inoculate 100 mL LB supplemented with kanamycin. Cells were grown at 37°C until the OD_{600} was between 0.5 and 0.7. After induction with 0.5 mM IPTG, the culture was grown overnight at 20°C. Cells were harvested by centrifugation at 2851 x g for 15 min. (4°C). Cell pellets were resuspended in 10 mL phosphate start buffer (50 mM Na_2HPO_4, 0.5 M NaCl, pH 7.5) (11) and lysed by sonication. Cell debris was removed by centrifugation (50 min. at 286,000 x g) and the protein was purified from the supernatant using gravity-flow chromatography with Ni-NTA Agarose (Qiagen). The protein was eluted with the phosphate start buffer containing 250 mM imidazole. Fractions were analyzed by 15% SDS-PAGE, Coomassie brilliant blue staining and Western blotting using an anti-His antibody (5 PRIME).

Peptidase treatment by LanTc39 on purified peptides. To reduce the high imidazole concentration in the purified His_6-LanA-SPN23F and His_6-LanTc39 samples, the buffer was replaced by 50 mM Na_2HPO_4 pH 7.5, 50 mM Na_2SO_4 (11) using Amicon® Ultra Centrifugal Filters (Millipore). The extracted His_6-LanA-SPN23F was purified from an *E. coli* strain also co-expressing either LanM-SPN23F or LanM-SPN23F/GdmD. A sample containing His_6-LanA-SPN23F (~0.1 mg/mL) and His_6-LanTc39 (~0.2 mg/mL) was incubated for three hours at 25°C in 50 mM Na_2HPO_4 pH 7.5, 50 mM Na_2SO_4 supplemented with 1 mM DTT (11). The reaction was stopped by chilling on ice and subsequently frozen at -20°C until further use. To analyze the peptide modification, samples were thawed and TFA was added until pH 4 was reached (with a minimum concentration of 0.1% TFA). Samples for MALDI-TOF MS analysis were prepared using C18 Zip-tips (Millipore) according to manufacturer protocol. MALDI-TOF mass spectrometry analysis was performed as described above.

Results

A putative lantibiotic encoded in the genome of *Streptococcus pneumoniae* ATCC 700669. BActeriocin GEnome mining tooL (BAGEL3) (15, 16) was used to scan the genome of *Streptococcus pneumoniae* ATCC 700669, and a putative typical class II lantibiotic gene cluster was identified (figure 1A).

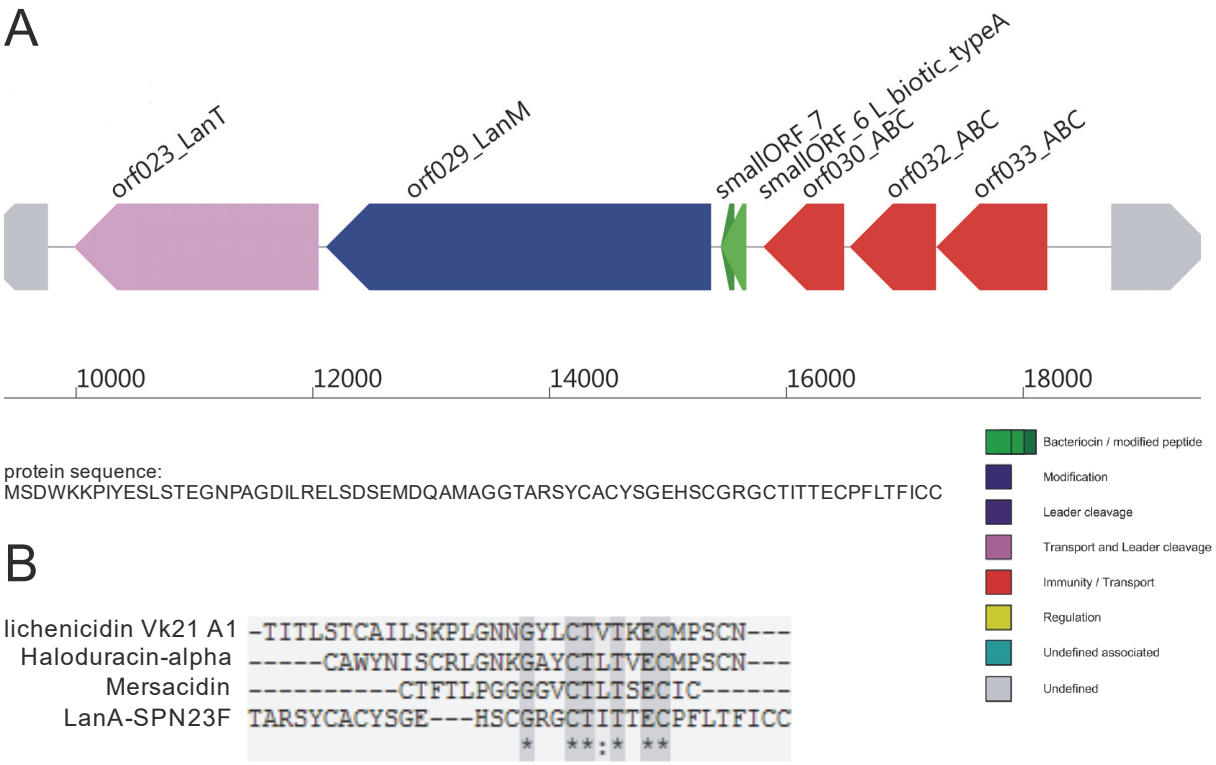

Figure 1: Putative class II lanthipeptide gene cluster from *Streptococcus pneumoniae* ATCC 700669. BAGEL3 analysis of the predicted class II lanthipeptide gene cluster (A). Alignment of mature LanA peptide encoding sequences of lichenicidin VK21 A1 (P86475), haloduracin-alpha (Q9KFM5), mersacidin (B4Y535) and the predicted LanA-SPN23F (B8ZK92) shows a conserved region for thioether formation (B).

The lantibiotic precursor gene was described previously by Croucher et. al. in 2009 (17) but not studied in detail. The two open reading frames (ORFs) downstream were identified as a lantibiotic synthetase (LanM) and a lantibiotic transporter (LanT). The three genes upstream of the lantibiotic precursor gene likely constitute an ABC transporter that is typically involved in self-immunity during lantibiotic production. In addition to the predicted bacteriocin gene clusters, BAGEL3 also uses the identified lantibiotic gene to scan it against bacteriocin databases. LanA-SPN23F shows homology with haloduracin-alpha (Q9KFM5) (18) with 37% identity, lichenicidin VK21 A1 (P86475) (19) with 39% identity and mersacidin (B4Y535) (20) with 35% identity. When the core peptide sequences of the above mentioned lantibiotics were aligned with the core peptide of LanA-SPN23F, a conserved region was found (figure 1B). This region is conserved throughout the mersacidin-like peptides and the α-peptides of the

two-component lantibiotics, suggesting a similar dehydration, including Thr72 and Thr74, and ring structure for His_6-LanA-SPN23F.

Co-expression of *Streptococcus pneumoniae* LanA-SPN23F and LanM-SPN23F in *E. coli*. In order to characterize the putative lantibiotic from *Streptococcus pneumoniae* Spain 23F-1, a His-tagged variant of LanA-SPN23F, His_6-LanA-SPN23F, was created. The gene was heterologously co-expressed in *E. coli* Bl21(DE3) cells together with the corresponding modification enzyme, LanM-SPN23F, using the pRSFDuet and pETDuet vectors. Although the expression was not evident on Coomassie stained Tris-tricine SDS-PAGE of whole cell lysates (figure 2A), cell free extract passed over an immobilized metal affinity chromatography (IMAC) column allowed the specific elution of a protein (~11kDa) that was absent in the empty vector control sample (figure 2C). The protein band could be detected by Western blotting using a Tetra-His antibody (5 prime), confirming the presence of a His-tagged LanA-SPN23F (figure 2D).

Analysis of His_6-LanA-SPN23F modifications. The purified His_6-LanA-SPN23F was subjected to reversed phase HPLC (RP-HPLC), monitored at a wavelength of 214 nm, and compared to an empty vector control sample. As shown in figure 3A, the sample containing His_6-LanA-SPN23F showed a unique peak around 28 minutes likely corresponding to the His_6-LanA-SPN23F. Next, MALDI-TOF MS analysis was used to validate the identity of the peptide and to determine the degree of modification by LanM-SPN23F. Dehydration of serine and threonine residues causes a mass loss of 18.02 Da, however, the cyclisation reaction that leads to the formation of the Lan or MeLan residues cannot be detected by MALDI-TOF as there is no mass change. The theoretical mass of the unmodified His_6-LanA-SPN23F is 9180.11 Da. Since the core peptide contains eight serine and threonine residues, a mass loss of 144.16 Da is expected for the completely modified peptide. A peak of 9031.72 Da (figure 4A) was identified, which closely matches the theoretical mass of eight times dehydrated His_6-LanA-SPN23F, i.e., 9035.95 Da, indicating complete dehydration.

In order to obtain a more precise insight in the degree of dehydration, His_6-LanA-SPN23F was submitted to trypsin digestion. Peptide fractions were separated using RP-HPLC and analyzed by MALDI-TOF MS. A peak of 2147.77 Da was identified as a peptide fragment containing the His-tag up to amino acid

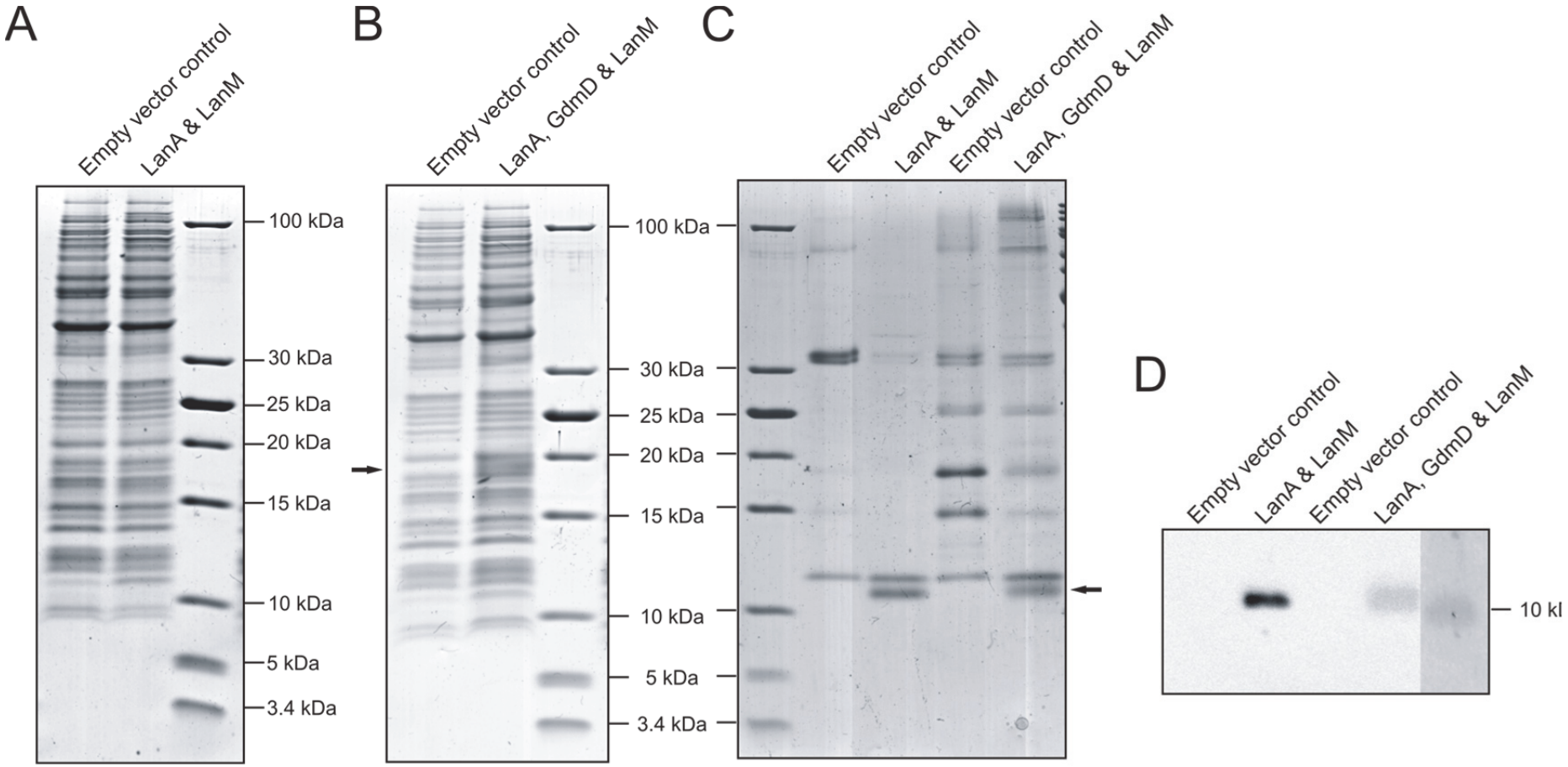

Figure 2: Tris-tricine Coomassie stained SDS-PAGE comparing cells of *E. coli* expressing empty vector or His_6-LanA-SPN23F and LanM-SPN23F (A) or empty vector or vectors harboring His_6-LanA-SPN23F, GdmD and LanM-SPN23F (B) (the arrow indicates the GdmD protein). The elution fractions of empty vectors, LanA-SPN23F & LanM-SPN23F and LanA-SPN23F, GdmD & LanM-SPN23F after IMAC purification showing a specific protein band about 11 kDa corresponding to His_6-LanA-SPN23F (C) (the arrow indicates His_6-LanA-SPN23F). Western blot developed with a Tetra-His antibody (5 prime) showing the presence of an 11 kDa protein (His_6-LanA-SPN23F) in the elution fractions after IMAC purification (D).

Lys19 (Gly2-Lys19) of the leader sequence (theoretical mass: 2146.90 Da, see also table 3). A second peak of 2061.28 Da was identified corresponding to amino acids Lys20-Arg38 of the leader peptide (theoretical mass: 2060.07 Da). In addition, two peaks of 4881.14 Da and 4864.47 Da were observed (figure 4C) that correspond to a seven and eight times dehydrated peptide including amino acid Glu39-Cys85 (the core peptide with a part of the leader peptide, theoretical mass: 4878.87 Da and 4860.85 Da, respectively). These results suggest that LanM-SPN23F is expressed and active in *E. coli* mediating the complete dehydration of His_6-LanA-SPN23F.

Introduction of decarboxylation modification in His_6-LanA-SPN23F. The three C-terminal amino acids of LanA-SPN23F are Ile-Cys-Cys, which matches the consensus sequence of the C-terminal amino acids for the recognition by EpiD which is [V/I/L/F/W/Y/(M)]-[A/S/V/T/C/(I/L)]-C (6). EpiD belongs to the group of LanD enzymes that are responsible for the formation of an S-aminovinyl-D-cysteine (AviCys) amino acid at the C-terminus of some LanA peptides. However, no LanD encoding gene was identified in the lantibiotic gene cluster of *Streptococcus pneumoniae* ATCC 700669. GdmD of *S. gallinarum* shows 87% identity with EpiD. In addition, this enzyme was shown to modify a nisin variant when heterologously co-expressed in *L. lactis* (7). Therefore, GdmD was chosen to determine if LanA-SPN23F is indeed a substrate of LanD and can be modified accordingly. The gene encoding GdmD was cloned in the pETD-lanM vector, and co-expressed with His_6-LanA-SPN23F and LanM-SPN23F in *E. coli* Bl21(DE3) cells. By SDS-PAGE a unique protein band of around 19 kDa corresponding to GdmD (figure 2B, indicated by the arrow) matching the molecular mass of 20,646 Da was observed. Cell free extract was passed over an IMAC column to extract the His_6-tagged peptide, and by Coomassie stained Tris-tricine SDS-PAGE and Western Blotting (figure 2C and D) the His_6-LanA-SPN23F could be detected. Next, the purified peptide was subjected to RP-HPLC and MALDI-TOF MS. As shown in figure 3, a unique peak around 27 minutes was evident on HPLC that was not observed in the empty vector control. Decarboxylation by GdmD will result in a mass loss of 46 Da due to the loss of a CO_2 molecule and two H atoms. The theoretical mass of eight times dehydrated

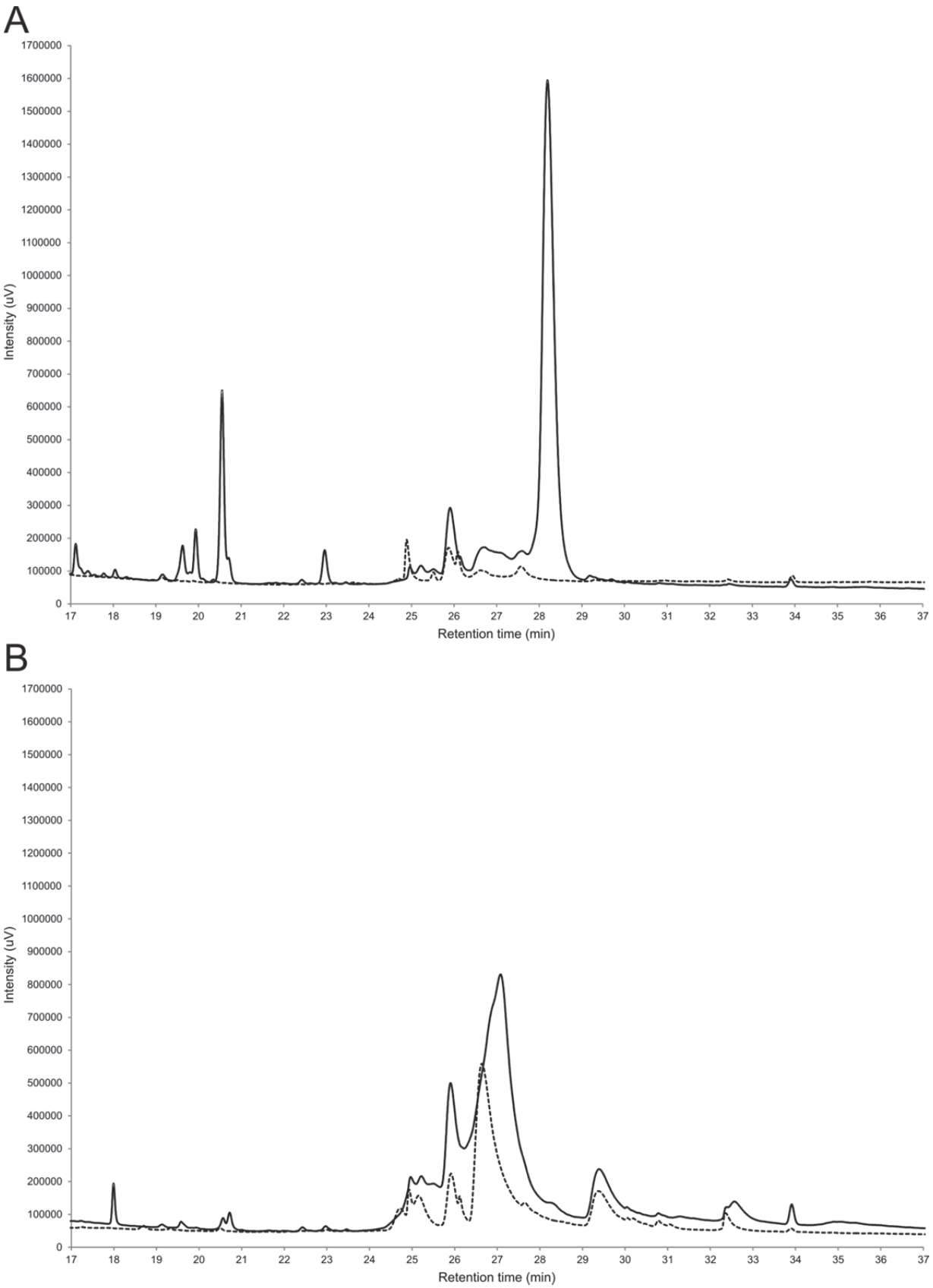

Figure 3: HPLC chromatograms of the produced LanA-SPN23F monitored at 214 nm. Comparison of the control (dotted line) and His_6-LanA-SPN23F containing sample (solid line) derived from an *E. coli* strain expressing His_6-LanA-SPN23F and LanM-SPN23F (A). Comparison of a control sample (dotted line) and a His_6-LanA-SPN23F containing sample (solid line) derived from an *E. coli* strain expressing His_6-LanA-SPN23F, GdmD and LanM-SPN23F (B).

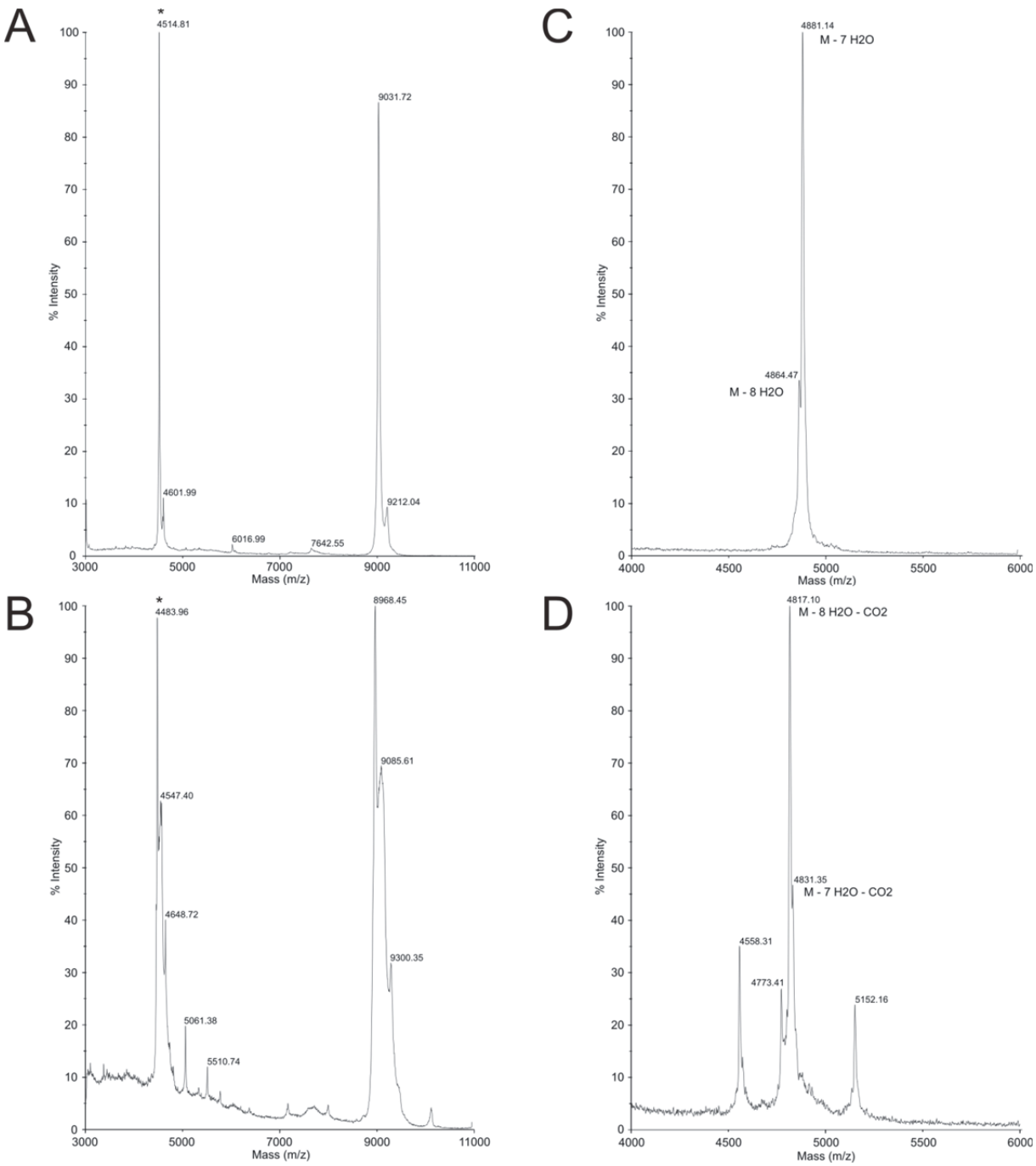

Figure 4: MALDI-TOF mass spectra of HPLC fractions containing His_6-LanA-SPN23F after co-expression with LanM-SPN23F (A) or LanM-SPN23F and GdmD (B). The asterisk (*) indicates a peak with the mass corresponding to double charged His_6-LanA-SPN23F. MALDI-TOF mass spectra of trypsin digested peptide fraction, Glu39-Cys85, of His_6-LanA-SPN23F after co-expression with LanM-SPN23F (C) or LanM-SPN23F and GdmD (D).

and decarboxylated His_6-LanA-SPN23F is 8989.95 Da. A peak with the mass of 8968.45 Da (figure 4B) that may correspond to the predicted modified His_6-LanA-SPN23F was observed. In addition, a peak of 9085.61 Da was observed

which may correspond to the seven times dehydrated and decarboxylated His_6-LanA-SPN23F. Since the difference between the theoretical mass and the observed mass is 20.5 Da the peptides were further analyzed by trypsin digestion and mass spectrometry to validate the modifications. Again peptide fragments corresponding to the parts of the leader peptide were found, i.e., Gly2-Lys19 with a mass of 2148.82 Da (theoretical mass of 2146.90 Da), and Lys20-Arg38 with a mass of 2060.92 Da (theoretical mass of 2060.07 Da). Importantly, peptides corresponding to amino acids Glu39-Cys85 with masses of 4817.10 and 4831.35 Da were observed (figure 4D). These correspond to the eight and seven times dehydrated and decarboxylated His_6-LanA-SPN23F (theoretical mass 4814.85 and 4832.87 Da, respectively), showing that His_6-LanA-SPN23F is indeed modified by both LanM-SPN23F and GdmD.

Leader peptide cleavage by peptidase domain LanTc39. Previous studies have shown that the isolated and purified peptidase domain of LctT can function in vitro to process the leader peptide from LctA and substrate variants to release a mature peptide (11). Our studies show that the LanA peptide under study can be fully modified with respect to dehydration and terminal cysteine modification, but to release an active peptide, the leader peptide needs to be removed. Therefore, the peptidase domain of the *S. pneumoniae* LanT-SPN23F was expressed as a His-tagged protein, termed His_6-LanTc39. The peptidase C39 domain of LanT-SPN23F covers the region of Val4-Phe131. Therefore, an extended fragment covering Leu1-Lys151 of LanT-SPN23F was cloned in the pRSFDuet vector. The protein was expressed in *E. coli* Bl21(DE3) cells, and detectable on SDS-PAGE as a 17 kDa protein (figure 5A) that also stains with a Tetra-His antibody (5 prime) in western blotting (figure 5B). To remove the leader of His_6-LanA-SPN23F it was incubated with His_6-LanTc39 and analyzed by MALDI-TOF mass spectrometry. The theoretical mass of unmodified and processed LanA-SPN23F is 3583.47 Da. However, no peaks were identified that could relate to the presence of processed, neither unmodified or modified, LanA-SPN23F.

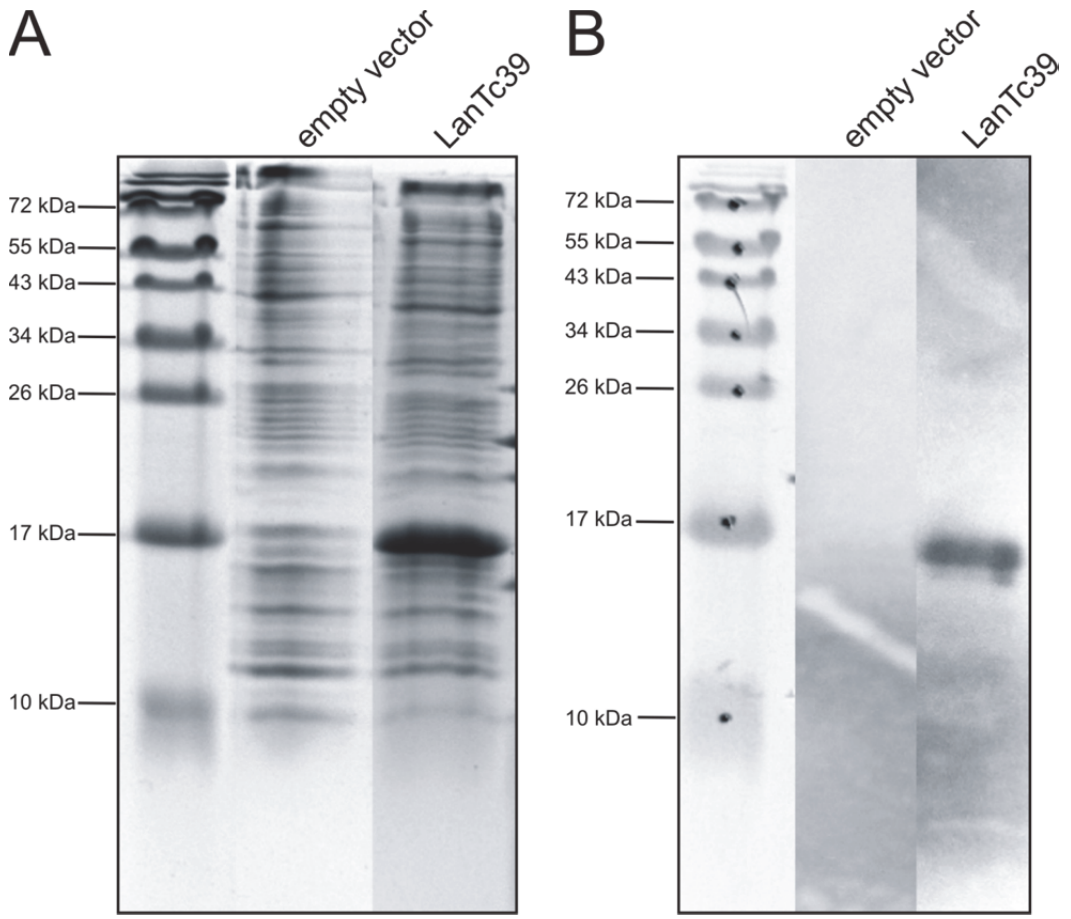

Figure 5: Coomassie stained SDS-PAGE comparing cells of *E. coli* expressing empty vector or His_6-LanTc39 (A). Western blot developed with a Tetra-His antibody (5 prime) showing the presence of His-tagged LanTc39 at a molecular mass of about 17 kDa (B). (The gel and blot figures are composites of samples analyzed on the same SDS-PAGE.)

Discussion

This study, examined the heterologous expression of a putative lantibiotic encoded in the genome of *Streptococcus pneumoniae* ATCC 700669. The gene was described previously and shown to be part of an autonomously mobile genetic entity (ICE), ICE*Sp*23FST81 (17). ICEs are transmitted between bacteria through conjugative transfer in a circularized form and insert into, and excise from, host DNA via site-specific recombination (17, 21). Via heterologous co-expression with the corresponding synthetase gene, the lanthipeptide could be detected. Unfortunately, expression appeared not high as purification and western blotting was necessary to detect the product. MALDI-TOF mass spectrometry analysis indicates that the peptide is fully dehydrated, namely eight

times, when produced in *E. coli*. This suggests that heterologously expressed LanM-SPN23F is active in *E. coli*. In addition, when adding GdmD to the expression system a fully dehydrated and decarboxylated His_6-LanA-SPN23F could be detected, suggesting LanA-SPN23F indeed is a substrate for a LanD enzyme although no such gene was found in the putative gene cluster. These results add to the construction of a generic class II lantibiotic system in *E. coli*. However, an active lantibiotic is obtained only after leader peptide cleavage. To realize this goal, the C39 peptidase domain of the LanT-SPN23F transporter was cloned and expressed. His_6-LanTc39 was readily obtained but co-incubation with His_6-LanA-SPN23F did not lead to the emerge of mature LanA-SPN23F peptides. This is, however, a preliminary finding. Since processing is a necessity before the biological function of LanA-SPN23F can be established, bioactivity of the heterologously produced LanA-SPN23F could not be demonstrated.

LanA-SPN23F is predicted by BAGEL3 and Croucher et. al. (17) to be a lantibiotic similar to lichenicidin A1 and mersacidin. Figure 1A shows a conserved region between lichenicidin A1, mersacidin, haladuracin A1 and LanA-SPN23F peptides. This region in lichenicidin A1 and mersacidin is associated with the third thioether ring that is thought to be most important for activity, inhibiting the peptidoglycan transglycosylation reaction as previously shown for mersacidin (17, 22). Such a lanthionine ring could also be formed in LanA-SPN23F. Trypsin digestion of the peptide occurs after lysine or arginine residue, except when the amino acid is followed by a proline. For that reason His_6-LanA-SPN23F should be digested into five fragments, Gly2-Lys19, Lys20-Arg38, Glu39-Arg55, Ser56-Arg69 and Gly70-Cys85. However, only three fragments were found by MALDI-TOF mass spectrometry. The first two fragments, Gly2-Lys19 and Lys20-Arg38, comprising the His-tag and a part of the leader peptide. The remaining three fragments are not found but instead one large fragment, Glu39-Cys85, was detected. Since the presence of a (methyl)lanthionine bond should protect the peptide against enzyme digestion, these data suggest that at least two (methyl)lanthionine rings were formed in the peptide. More detailed studies need to be performed to resolve the exact ring structure formed by LanM-SPN23F in His_6-LanA-SPN23F. In addition, bioactivity of LanA-SPN23F could give indication if the (methyl)lanthionine rings are correctly formed. Unfortunately, leader cleavage was unsuccessful and therefore antimicrobial activity of LanA-SPN23F could not be determined.

Summarizing, these data demonstrate that *E. coli* can be used for the production of class II lantibiotics as exemplified by the expression and post-translational modification of a previously uncharacterized lanthipeptide from *Streptococcus pneumoniae*.

Acknowledgements

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Table 3: Peptide sequence of His-LanA-SPN23F, peptide fraction determined after trypsin digestion and their theoretical mass.

	His-tag sequence	Lantibiotic leader peptide	Lantibiotic core peptide	Mass (Da)
Full protein				
	1 5 10	15 20 25 30 35 40 45 50	55 60 65 70 75 80 85	
His_6-LanA	MGSSHHHHHHSQDP	MSDWKKPIYESLSTEGNPAGDILRELSDSEMDQAMAGG	TARSYCACYSGEHSCGRGCTITTECPFLIFICC	9180.11*
Peptide fragments (after trypsin digestion)				
	1 14	15 19		
Gly2-Lys19	MGSSHHHHHHSQDP	MSDWK		2146.90**
		20 38		
Lys20-Arg38		KPIYESLSTEGNPAGDILR		2060.07**
		39 52	53 85	
Glu39-Cys85		ELSDSEMDQAMAGG	TARSYCACYSGEHSCGRGCTITTECPFLIFICC	5005.01**

* The average mass of the protein, $(M+H)^+$, calculated without the initial methionine.
** Monoisotopic mass of the peptides, $(M+H)^+$, was calculated without the initial methionine.
TS Ser or Thr residues that may be dehydrated by LanM-SPN23F

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Chapter 7

Summary and concluding remarks

Lantibiotics, lanthionine-containing ribosomally produced peptides, possess antimicrobial activity and some even display activity towards drug resistant pathogens, like methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant enterococci (VRE) (1-3). In addition, lanthipeptides can possess morphogenetic (4), antiviral (5) or anti-allodynic (6) activity. The presence of the thioether bridge and other post translational modifications provide lanthipeptides with a higher protection against proteolytic degradation. This makes lantibiotics and lanthipeptides interesting targets for the use as therapeutic agents. However, there are also several challenges that need to be overcome. Many lantibiotics are liable to degradation by enzymes present in the gastrointestinal tract, therefore, applications of lantibiotics has been limited to topical applications only. In addition, lantibiotics are often not sufficiently stable over the full pH range. Nisin, for example, looses activity above pH 7 since under basic conditions oxidation of the (methyl)lanthionine bridges occurs resulting in a loss of the antimicrobial activity (7).

The use of lanthipeptide biosynthesis enzymes in bioengineering to introduce (methyl)lanthionine residues or additional modifications in peptides is of particular interest as it provides a means to generate an even greater diversity of lanthipeptides and a potential methodology to tackle the aforementioned challenges. Therefore, this thesis focused on creating a generic system for the production of class II lantibiotics/lanthipeptides. Unlike class I lanthipeptides, which have an elongated structure, class II lanthipeptides have a more globular structure and require the activity of an integrated dehydratase/cyclase enzyme for post-translational modification. Therefore, the intended production system will contain a known class II lantibiotic LanM modification enzyme and possibly the associated transporter (figure 1).

Initially, in our studies the Gram-positive host strain *Lactococcus lactis* NZ9000 was explored as a production host employing the modification enzymes (LtnM1 or LtnM2) and transporter (LtnT) of lacticin 3147. Lacticin 3147 is a two-

component lantibiotic of wich the LtnA1 peptide is considered an typical example of a class II lanthipeptide. Co-expression of LtnA1, LtnM1 and LtnT or LtnA2, LtnM2 and LtnT led to the production of modified, processed and active lacticin 3147 (chapter 3). In addition, Kuipers et al. (8) showed that LtnM2 and LtnT are able to modify and transport non-lantibiotic peptides fused behind the LtnA2 leader peptide, namely angiotensin variants which suggested a relaxed substrate specificity for LtnM2. This drove further exploration of the substrate specificities of LtnM1 and LtnM2 as well as generic aspects of the production system. The respective enzymes where co-expressed with substrate variants (chapter 4). Although, Cys-less peptide variants of LtnA1 and LtnA2 where produced to some extent when co-expressed with LtnT/LtnM2, production levels were very low which interfered with the reproducibility. In addition, most peptide variants generated could not be detected. These results suggest some degree of substrate specificity of the LtnM enzymes, but the in general low production of peptide variants makes this system less suitable for a generic production system. In this regard, the gene cluster of lacticin 3147 already indicates substrate specificity, as it encodes two separate dedicated modification enzymes specific for the two peptides.

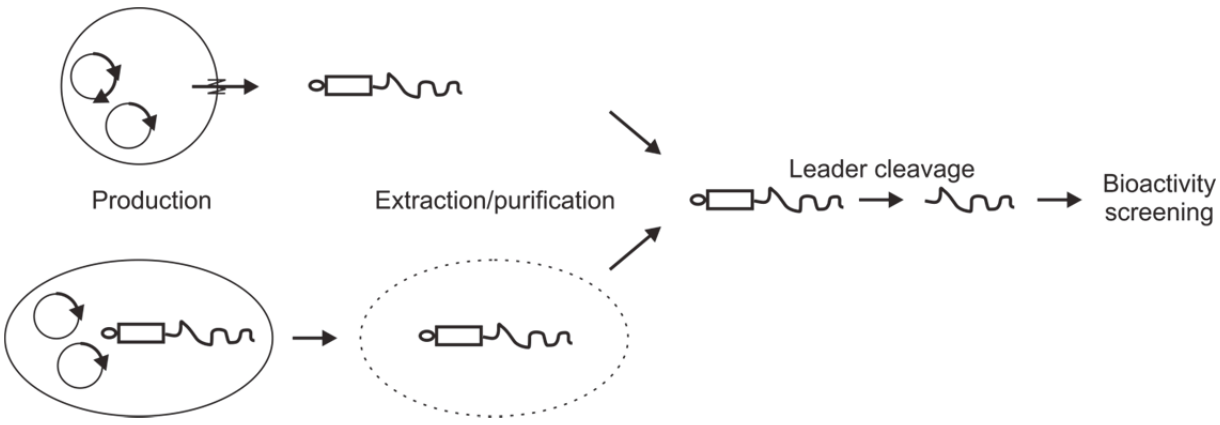

Figure 1: Schematic representation of the intended generic production systems. Expressing a known modification enzyme (LanM), possibly a lanthipeptide transporter (LanT) and the lanthipeptide substrate (LanA) in a Gram-positive host strain such as *L. lactis* (top). In this system, the product is secreted in an unmatured form. Alternatively, a Gram-negative host strain like *E. coli* (bottom) expresses only the LanM and LanA and the product accumulates intracellularly. After induced expression and production, the peptide needs to be extracted and purified, where up leader cleavage by a LanP domain, or alternative protease should result in the release of the matured lanthipeptide.

The lacticin 3147 transporter used in the production system also exhibited some degree of substrate specificity. Transport of unmodified peptides is possible but not favorable (chapter 2), Cys-less peptides are transported to some extent, but when the core peptides were swapped, production was completely abolished (chapter 4). Class II lantibiotic transporters, like LtnT, belong to the ABC transporter maturation and secretion (AMS) protein family, meaning they remove the leader peptide during transport thus secreting the matured form of the lantibiotic. However, in a generic production system the production of a mature and active uncharacterized lantibiotic is not desired since it might display bioactivity towards the production host. Removing the N-terminal C39 peptidase domain of LtnT or mutating the catalytic cysteine in this peptidase domain resulted in a non-functioning LtnT (chapter 2). This suggests that an active peptidase domain is required for transport, confirming earlier studies for NukT, the transporter of nukacin ISK-1 (9). As an alternative means, one can envision the secretion of the non-maturated lanthipeptide, for instance by replacing the double glycine motif of the leader peptide with a factor Xa proteases site. This would result in the production of the inactive peptide that next might be converted into the active form upon processing by the factor Xa protease. Indeed, NisB/NisC and NisT accept the presence of a factor Xa site in nisin and pre-nisin can be activated by the post-secretion treatment with factor Xa (10). However, by replacing the double glycine motif or placing the factor Xa between the double glycine motif and the core peptide of LtnA1, peptide production was completely eliminated (chapter 4). Although the exact reason of this defect was not further analyzed, it appears that such modifications prevent transport.

To circumvent the low production levels and the export problems, the Gram-negative host strain *Escherichia coli* was examined as a potential generic production system for intracellular lanthipeptides. Heterologous expression of lanthipeptides in *E. coli* has been shown before for nukacin ISK-1, nisin, prochlorosins and haloduracin (11, 12). The anticipated production system was based on either the modification enzymes MutM or LtnM2 and these enzymes were co-expressed with the corresponding his-tagged substrate peptide (chapter 5). In these cases fully dehydrated preMutA and preLtnA2 could be isolated from the cytosolic fraction of *E. coli* cells. Although, fully dehydrated peptides were detected also incomplete dehydrated intermediates were identified, suggesting

that the heterologous expression in *E. coli* is possible but not optimal. Interestingly, MutM displayed a relaxed substrate specificity towards various core peptides of LtnA1, LtnA2 and even the class I lanthipeptide nisin when fused behind the MutA leader peptide. Although dehydration took place, the extent of dehydration varies from full to under dehydration. While LtnM2 in conjunction with LtnT seemed to display a higher degree of substrate specificity when expressed in *L. lactis*, the intracellular expression in *E. coli* appeared to be equipped with a less stringent substrate specificity. This study only examined the dehydration and did not establish whether the correct thioether bridges were formed. In this respect, for none of the heterologously expressed lanthipeptides, antimicrobial activity could be demonstrated since the peptides were produced as precursor peptides. For future research it will be interesting to introduce a protease site in the substrate peptide so the peptide can be matured in vitro. Although, introduction of the factor Xa protease site in LtnA1 in the Gram-positive host production system (chapter 4) resulted in production loss, it exhibited promising results in a NisB/NisC/NisT expression system (10). In addition, a study by Patton et al. (13) showed that mutating the double glycine motif of the lacticin 481 leader peptide did not result in loss of LctM activity. Introduction of the factor Xa protease site could be a promising addition for the production system in the heterologous expression host *E. coli*.

The results gathered in chapter 2 to 5 suggest that the modification enzymes of class II lantibiotics are highly specific. When mutations are made in the substrate peptides, in most cases the production level dropped or production was even completely abolished. In addition, incomplete modification occurred and the reproducibility was low. Due to this presumed substrate specificity it turned out to be difficult to set up a generic production system in *L. lactis*. In contrast, heterologous expression in *E. coli* of lanthipeptides with their corresponding enzymes has been shown before (chapter 5, (11, 12)) and provides an alternative for a generic production system. To examine this possibility, a putative class II lantibiotic gene cluster in *Streptococcus pneumoniae* ATCC 700669 was identified with BAGEL3. The genes encoding the peptide and the biosynthesis enzyme were heterologously expressed in *E. coli* (chapter 6). The core peptide of LanA-SPN23F contains eight serine and threonine residues, and MALDI-TOF mass spectrometry analysis of the

heterologously produced peptide indeed indicate seven to eight times dehydration events. In addition, an extra modification, namely the decarboxylation of the carboxyl-terminus by GdmD, could be introduced. GdmD was found to be active in *E. coli* and able to modify the heterologously expressed LanA-SPN23F. In order to obtain the active peptide an attempt was made to cleave the leader peptide by heterologously expressing the N-terminal C39 peptidase domain of the corresponding LanT in *E. coli*. Although, the peptidase domain was expressed, cleavage activity could not be established. Nevertheless, the use of such a peptidase domain should be further considered as a means to process heterologously produced class II lanthipeptides. As Furgerson Ihnken et al. (14) demonstrated in vitro activity of the lacticin 481 LtnT C39 peptidase domain by cleaving the lacticin 481 leader peptide and rendering a mature lacticin 481.

Overall, the use of *E. coli* as a generic production system for class II lanthipeptides shows a number of promising features but the system should be further examined on its promiscuity. For future research on the production of novel lantibiotics identified in metagenomes, the heterologous expression of the required part of a given gene cluster seems, however, a more reliable approach. This would then concern expression of the substrate peptide together with their natural modification enzyme. The availability of commercial DNA synthesis at lower prices and improved quality of large DNA fragments might convert this approach even into a high throughput methodology. This will be a necessity for the metagenome-wide identification of novel antimicrobials. This system could be complemented by the addition of extra modification enzymes when required. For instance, the decarboxylase GdmD showed a less stringent substrate specificity in this study. Finally, the active peptide needs to be produced. This requires an efficient means of post-production processing. The use of the isolated peptidase domain of the LanT transporter might provide such an enzyme but further work is needed to define its substrate specificity. Also, introduction of a protease cleavage site is a possible solution so in vitro the lanthipeptide can be converted into an active mature form. Only when all these requirements are met, it becomes feasible to explore metagenomes for unknown lanthipeptides as potential new antimicrobials.

Table 1: Summary of detected peptides in this thesis, by means of MALDI-TOF mass spectrometry, in supernatant of *L. lactis* NZ9000 or cell free extract of *E. coli* co-expressing transporter and/or modification enzymes in combination with the indicated substrate variants.

	***Lactococcus lactis* host**		
	LtnT	**LtnM1/LtnT**	**LtnM2/LtnT**
Wild type peptides			
LtnA1	0x Dha/Dhb	7x Dha/Dhb	X
LtnA2	0x Dha/Dhb	X	8x Dha/Dhb
Fusion peptides			
LtnA1-A2	X	X	X
LtnA2-A1	X	X	X
Cysteine less peptides			
LtnA1(C-less)	0x Dha/Dhb	X	N/A
LtnA2(C-less)	X	N/A	X
LtnA1-A2(C-less)	X	X	N/A
LtnA2-A1(C-less)	0x Dha/Dhb	N/A	0x Dha/Dhb
Factor Xa containing peptides			
LtnA1-xa1	X	X	N/A
LtnA1-xa2	X	X	N/A

	***Escherichia coli* host**			
	LtnM2	**MutM**	**LanM-SPN23F**	**LanM-SPN23F/ GdmD**
Wild type peptides				
His_6-LtnA2	0-8x Dha/Dhb	N/A	N/A	N/A
His_6-MutA	N/A	0-4x Dha/Dhb	N/A	N/A
His_6-LanA-Spn23F	N/A	N/A	7, 8x Dha/Dhb	7, 8x Dha/Dhb+ decarboxylation
Fusion peptides				
His_6-LtnA2-NisA	3x Dha/Dhb	N/A	N/A	N/A
His_6-Mut-LtnA1	N/A	2-4x Dha/Dhb	N/A	N/A
His_6-Mut-NisA	N/A	1, 4x Dha/Dhb	N/A	N/A
His_6-Mut-ltnA2	N/A	2, 7-8x Dha/Dhb	N/A	N/A

1x Dha/Dhb amount of dehydrations detected for the peptide by MALDI-TOF MS
X no peptide detected by MALDI-TOF MS
N/A not applicable/not determined

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Chapter 8

Samenvatting en concluderende opmerkingen

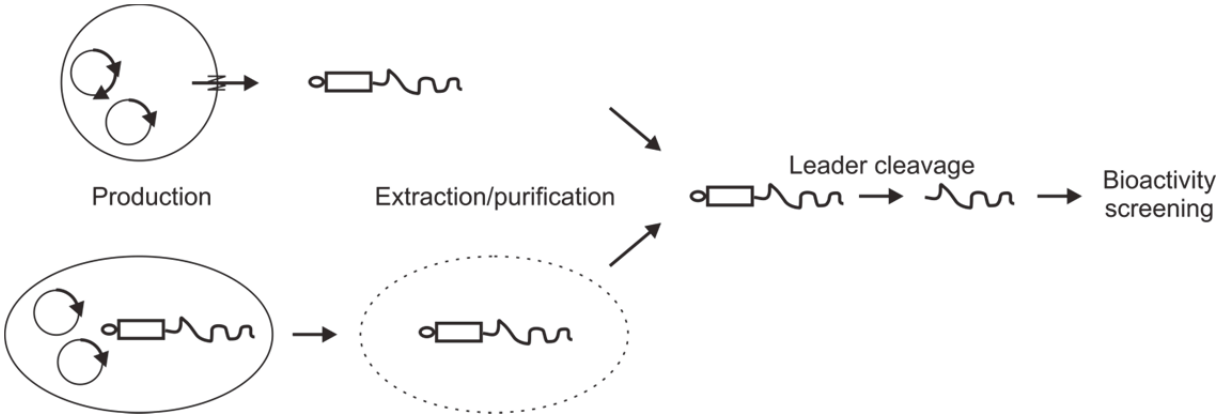
Infectieziekten, veroorzaakt door pathogene micro-organismen, worden over het algemeen behandeld door middel van antibiotica. Maar helaas zijn er tegenwoordig steeds meer antibioticaresistente bacteriën die niet meer behandeld kunnen worden met traditionele antibiotica. Daarom is het noodzakelijk om nieuwe soorten antibiotica te ontwikkelen om de resistentie te omzeilen. Lantibiotica zouden in aanmerking kunnen komen als een nieuw soort antibiotica. Het hier beschreven onderzoek richt zich daarom op de productie van lantibiotica.

Lanthipeptiden zijn een groep van lanthionine bevattende ribosomaal geproduceerde peptiden, wanneer deze peptide antimicrobiële activiteit bezitten worden ze lantibiotica genoemd. Deze peptiden kunnen activiteit vertonen tegen antibioticaresistente pathogene micro-organismen, zoals methicilline-resistente *Staphylococcus aureus* (MRSA) en vancomycine-resistente enterococci (VRE) (1-3). Lanthipeptiden kunnen ook morfogenetische (4), antivirale (5) of anti-allodynische (6) activiteit bezitten. De aanwezigheid van de kenmerkende thio-etherbruggen en andere post-translationale modificaties zorgen ervoor dat de lanthipeptiden beter beschermd zijn tegen proteolytische afbraak. Deze eigenschap maken lantibiotica en lanthipeptiden zeer interessant voor het gebruik als therapeutische stoffen.

Echter bezitten lantibiotica ook een aantal niet-gewenste eigenschappen die eerst overwonnen moeten worden alvorens medische toepassingen mogelijk zijn. Zo kunnen lantibiotica gevoelig zijn voor afbraak door enzymen die aanwezig zijn in het maagdarmkanaal. Daardoor is het gebruik van deze peptiden beperkt tot toepassingen op oppervlaktes zoals de huid, waarbij ze zijn verwerkt in een zalf. Daarnaast zijn lantibiotica vaak niet stabiel bij veranderende pH waarden. Nisine, een lantibioticum, verliest bijvoorbeeld zijn antimicrobiële activiteit wanneer de pH stijgt, omdat onder alkalische condities oxidatie van de (methyl)lanthionine verbindingen plaats kan vinden (7).

Het gebruik van de biosynthese enzymen van lanthipeptiden in bioengineering, om zo (methyl)lanthionine verbindingen of aanvullende

modificaties in peptiden aan te brengen, zou een mogelijke methode kunnen zijn om een nog grotere verscheidenheid aan lanthipeptiden te creëren. Met een grotere diversiteit aan lanthipeptiden kunnen ook nieuwe lantibiotica ontwikkeld worden die eventueel de bovengenoemde problemen aanpakken. Het onderzoek beschreven in dit proefschrift is daarom gericht op de ontwikkeling van een generiek systeem voor de productie van klasse II lantibiotica/lanthipeptiden. In tegenstelling tot klasse I lantibiotica, welke langgerekte structuren vormen, bezitten klasse II lantibiotica een meer globulaire (bolvormige) structuur. In klasse II lantibiotica worden de karakteristieke modificaties niet aangebracht door twee aparte enzymen maar door één enkele enzym dat zowel dehydratase als cyclase activiteit bezit. Het beoogde productiesysteem zal bestaan uit een bekend klasse II lantibiotica modificatie-enzym, LanM, en eventueel het bijbehorende transport enzym (figuur 1) om het product uit te kunnen scheiden.



Figuur 1: Schematische weergave van de beoogde generieke productiesystemen. Expressie van een bekend modificatie enzym (LanM), eventueel de transporter (LanT) en het substraat (LanA) in een Grampositieve stam zoals *Lactococcus lactis* (boven). In dit systeem wordt het product uitgescheiden als niet actief pre-lantibiotica. Als alternatief, expressie van het modificatie enzym in een Gramnegatieve gastheer zoals *Escherichia coli* (onder). Hier worden alleen het LanM enzym en LanA substraat tot expressie gebracht, en zal het product intracellulair ophopen. Na geïnduceerde expressie en productie van het peptide zal deze geëxtraheerd en gezuiverd worden. Daarna word het leader peptide verwijderd doormiddel van een LanP domein of een protease. Dit laatste zal resulteren in het vrijkomen van het mature lanthipeptide.

De Grampositieve *Lactococcus lactis* NZ9000 stam is in eerste instantie geanalyseerd als mogelijke productie stam door in dit organisme de modificatie

enzymen (LtnM1 of LtnM2) en transporter (LtnT) van lacticine 3147 tot expressie te brengen. Lacticine 3147 is een twee-componenten lantibioticum, en bestaat uit twee peptiden die samen een actief lantibioticum vormen. Van de twee peptiden lijkt het LtnA1 peptide het meest op een klasse II lantibioticum. Co-expressie van de combinatie LtnA1, LtnM1 en LtnT of LtnA2, LtnM2 en LtnT resulteerde in gemodificeerd en actief lacticine 3147 (hoofdstuk 3). Kuipers et al. (8) liet al eerder zien dat LtnM2 en LtnT niet-lantibioticum peptiden, zoals angiotensine varianten, kunnen modificeren en transporteren. Dit suggereert dat LtnM2 een brede substraat specificiteit bezit. Zodoende is er voor gekozen om de substraat specificiteit van LtnM1 en LtnM2 en hun generieke toepassing in het productiesysteem verder te onderzoeken. LtnM1 en LtnM2 werden tot expressie gebracht samen met verschillende substraat varianten (hoofdstuk 4). Hierbij zijn onder meer de cysteïne residuen vervangen door alanine residuen om te voorkomen dat er thio-etherbruggen gevormd kunnen worden. Nadat een aantal Cys-loze peptide varianten van LtnA1 en LtnA2 werden geproduceerd wanneer ze tot co-expressie waren gebracht met LtnT/LtnM2, bleek dat het productie niveau over het algemeen dusdanig laag was dat dit de reproduceerbaarheid van het resultaat belemmerde. Tevens konden de meeste peptide varianten niet worden gedetecteerd. De gevonden resultaten suggereren dat de LtnM enzymen toch enige substraat specificiteit bezitten. De organisatie van het lacticine 3147 genencluster geeft zelf al een indicatie dat er sprake is van substraat specificiteit. Het genencluster bevat namelijk twee genen die coderen voor twee aparte gespecialiseerde modificatie enzymen voor de twee lacticine 3147 peptiden. Er lijkt dus sprake van specialisatie. Echter, het feit dat over het algemeen de productie van de peptide varianten laag was maakt dit systeem minder geschikt als een generiek productiesysteem.

Ook de lacticine 3147 transporter die gebruikt is in het productiesysteem lijkt een zekere mate van substraat specificiteit te tonen. Transport van het niet-gemodificeerde peptide is mogelijk maar lijkt inefficiënt (hoofdstuk 2). De Cys-loze peptiden worden maar deels getransporteerd en wanneer de kernpeptiden achter de leader peptiden worden verwisseld stopt de productie (hoofdstuk 4).

Klasse II lantibiotica transporters, zoals LtnT, behoren tot de familie van de ABC transporter maturatie en secretie (ABC transporter maturation and secretion (AMS)) eiwitten. Deze eiwitten verwijderen het leader peptide van het

lantibioticum tijdens het transport over het membraan en scheiden zo het mature lantibioticum uit. Deze eigenschap, om het mature en actieve niet-gekarakteriseerde lantibioticum te produceren, is in het generieke productiesysteem niet gewenst. Het zou eventueel kunnen dat het geproduceerde lantibioticum bio-activiteit vertoont tegen de productie stam. Om de productie van actieve lantibiotica tegen te gaan is er getracht de peptidase activiteit van de LtnT transporter te verwijderen. Het verwijderen van de N-terminale C39 peptidase domein of het muteren van het katalytische cysteïne residue van dit domein resulteerde in een niet werkend LtnT (hoofdstuk 2). Deze resultaten suggereren dat LtnT een actief peptidase domein nodig heeft om het transport van het lantibioticum te realiseren. Mogelijk zijn beide processen gekoppeld. Eerdere studies voor NukT, het transporteiwit van nukacine ISK-1, gaf vergelijkbare resultaten (9).

Een alternatieve methode voor het uitscheiden van het niet-mature lanthipeptide is het vervangen van het dubbele glycine motief van het leader peptide met bijvoorbeeld een specifieke factor Xa protease sequentie. Het geproduceerde peptide zou dan in een vervolgstap, door middel van het factor Xa protease, omgezet kunnen worden naar zijn actieve vorm. De enzymen NisB en NisC accepteren de factor Xa sequentie in pre-nisine dat vervolgens kan worden omgezet naar het actieve nisine door middel van de behandeling met factor Xa (10). Echter na het vervangen van het dubbele glycine motief of het plaatsen van de factor Xa sequentie tussen het dubbele glycine motief en het kernpeptide van LtnA1, werd de productie volledig geëlimineerd (hoofdstuk 4). Alhoewel de exacte reden van dit defect niet verder geanalyseerd is, geeft het wel aan dit soort modificaties een negatief effect hebben op de productie en mogelijk het transport van de peptiden.

Vanwege de lage productie niveaus en de problemen met uitscheiding in *L. lactis*, is de Gramnegatieve gastheer *Escherichia coli* geanalyseerd als potentieel generiek productiesysteem voor intracellulaire lanthipeptiden. Heterologe expressie van lanthipeptiden in *E. coli* is al aangetoond voor nukacine ISK-1, nisine, prochlorosinen en haloduracine (11, 12). In dit geval was het productiesysteem gebaseerd op het modificatie enzym MutM of LtnM2, waarna deze enzymen tot expressie zijn gebracht met hun overeenkomstige hexahistidine-gelabelde substraat peptide (hoofdstuk 5). Volledig

gedehydrateerd preMutA en preLtnA2 konden geïsoleerd worden uit de cytosolische fractie van *E. coli* cellen. Daarnaast werden ook incompleet gedehydrateerde tussenvormen geïdentificeerd. Dit suggereert dat de heterologe expressie in *E. coli* mogelijk is maar nog niet optimaal. Het enzym MutM liet een ogenschijnlijke bredere substraat specificiteit zien. Verschillende kernpeptiden van bijvoorbeeld LtnA1, LtnA2 en zelfs de klasse I lanthipeptide nisine werden gedehydrateerd wanneer deze gefuseerd waren achter het MutA leader peptide. Hoewel dehydratatie plaats vond varieerde de dehydratie van volledig tot onvolledig. In het *L. lactis* systeem gaf LtnM2 in combinatie met LtnT ogenschijnlijk een hogere mate van substraat specificiteit in vergelijking tot de lagere substraat specificiteit in het intracellulaire *E. coli* systeem. Mogelijk speelt de substraatspecificiteit van het transporteiwit hierin een rol.

In deze studie is alleen dehydratie aangetoond en is niet onderzocht of de correcte thio-etherbruggen worden gevormd. Omdat de peptiden als prepeptiden geproduceerd werden met het *E. coli* systeem, kon de antimicrobiële activiteit voor de heteroloog geproduceerde lanthipeptiden niet bepaald worden. Voor toekomstig onderzoek is het daarom van belang om een protease herkenningssequentie te introduceren in het prepeptide zodat in vitro het mature peptide gegenereerd kan worden. Ondanks dat de introductie van de factor Xa protease sequentie in het Grampositieve klasse II lanthipeptide productiesysteem (hoofdstuk 4) resulteerde in verlies van productie liet een studie met het NisB/NisC/NisT expressiesysteem met een overeenkomstige benadering goede resultaten zien (10). Tevens is eerder gevonden dat mutaties in de dubbele glycine motief van lacticine 481 niet interfereren met de werking van het modificatie enzym LctM (13). Al met al kan de introductie van de factor Xa protease sequentie een veelbelovende toevoeging in het heterologe *E. coli* productiesysteem zijn. Een alternatief is het gebruik van het protease domein van de transporter dat, indien actief, toegepast kan worden om het prepeptide op een juiste wijze om te zetten naar het mature lanthipeptide.

De resultaten in hoofdstuk 2 tot en met 5 suggereren dat de modificatie enzymen van klasse II lantibiotica een hoge specificiteit bezitten. Wanneer er mutaties gemaakt worden in de substraat peptiden daalt in veel van de gevallen het productie niveau of stopt de productie zelfs compleet. Daarbij vindt veelal

onvolledige modificatie plaats en bleek de reproduceerbaarheid laag. Door deze veronderstelde substraatspecificiteit bleek het moeilijk te zijn om een generiek productiesysteem in *L. lactis* op te zetten. Daarentegen is de heterologe expressie van lanthipeptiden en bijbehorende enzymen in *E. coli* mogelijk (hoofdstuk 5, (11, 12)). Om dit verder te onderzoeken is een mogelijke klasse II lantibioticum genencluster met behulp van BAGEL3 geïdentificeerd in *Streptococcus pneumoniae* ATCC 700669. Vervolgens zijn de genen coderend voor het biosynthese enzym en het substraat heteroloog tot expressie gebracht in *E. coli* (hoofdstuk 6). Het kernpeptide van LanA-SPN23F bevat acht serine en threonine residuen die eventueel gemodificeerd kunnen worden door LanM-SPN23F. MALDI-TOF massaspectrometrie analyse van het heteroloog geproduceerde peptide toonde inderdaad aan dat het peptide zeven tot acht keer werd gedehydrateerd. Daarnaast is een extra modificatie toegevoegd, namelijk de decarboxylatie van het carboxyl-einde van het peptide door GdmD. GdmD bleek actief in *E. coli* en kon het heteroloog tot expressie gebrachte LanA-SPN23F decarboxyleren.

Om actief peptide te verkrijgen werd een poging gedaan om het leader peptide te verwijderen door middel van het heteroloog tot expressie gebrachte N-terminale C39 peptidase domein van LanT-SPN23F. Hoewel dit domein tot expressie kon worden gebracht in *E. coli* kon er geen activiteit worden vastgesteld. Niettemin is de toepassing van dergelijke peptidase domeinen een interessante methode om heteroloog geproduceerde klasse II lanthipeptiden te activeren. Zo lieten Furgerson Ihnken e.a. (14) in vitro activiteit van het lacticine 481 LtnT C39 peptidase domein zien, waarbij het mature lacticine 481 werd gevormd na afsplitsing van het lacticine 481 leader peptide.

Kortom, het gebruik van *E. coli* als generiek productiesysteem voor klasse II lanthipeptiden toont een aantal veelbelovende eigenschappen. Echter het systeem moet verder onderzocht worden op brede toepassing. Voor toekomstig onderzoek lijkt de meest betrouwbare methode voor de succesvolle productie van niet eerder gekarakteriseerde lantibiotica, de heterologe expressie van het gewenste gedeelte van een genencluster. Dit zou dan de expressie van het substraat peptide tezamen met het bijbehorende natuurlijke modificatie enzym betreffen. Door de toenemende beschikbaarheid van commerciële DNA-synthese tegen lage prijzen met een betere kwaliteit van grote DNA-fragmenten kan men

deze aanpak zelfs omzetten in een high throughput methode. Dit is noodzakelijk voor metagenoom-brede identificatie van nieuwe antimicrobiële stoffen. Dit productiesysteem kan wanneer nodig worden aangevuld met extra modificatie enzymen. Het decarboxylase GdmD laat in deze studie een minder stringente substraatspecificiteit zien en is daarom een mogelijke kandidaat om toe te voegen aan het productiesysteem. Uiteindelijk zal een actief peptide moeten worden geproduceerd. Dit houd in dat er een efficiënte manier voor de vorming van het actieve lanthipeptide moet worden ontwikkeld. Het gebruik van een geïsoleerd peptidase domein van LanT transporters zou een manier kunnen zijn, maar verder onderzoek is nodig om de toepasbaarheid en de substraat specificiteit te bepalen. Alleen wanneer aan al deze eisen wordt voldaan zal het mogelijk worden om metagenomen te verkennen voor onbekende lanthipeptiden met potentiële antimicrobiële werking.

Tabel 1: Samenvatting van de in dit proefschrift gedetecteerde peptiden middels MALDI-TOF massaspectrometrie in het supernatant van *L. lactis* NZ9000 of het cel-vrij extract van *E. coli* in welke de transporter en/of de modificatie-enzymen in combinatie met het aangegeven substraat varianten tot co-expressie zijn gebracht.

	***Lactococcus lactis* gastheer**			
	LtnT	**LtnM1/LtnT**	**LtnM2/LtnT**	
Wild-type peptiden				
LtnA1	0x Dha/Dhb	7x Dha/Dhb	X	
LtnA2	0x Dha/Dhb	X	8x Dha/Dhb	
Fusie peptiden				
LtnA1-A2	X	X	X	
LtnA2-A1	X	X	X	
Cysteine loze peptiden				
LtnA1(C-loos)	0x Dha/Dhb	X	N/A	
LtnA2(C-loos)	X	N/A	X	
LtnA1-A2(C-loos)	X	X	N/A	
LtnA2-A1(C-loos)	0x Dha/Dhb	N/A	0x Dha/Dhb	
Factor Xa bevattende peptiden				
LtnA1-xa1	X	X	N/A	
LtnA1-xa2	X	X	N/A	
	***Escherichia coli* gastheer**			
	LtnM2	**MutM**	**LanM-SPN23F**	**LanM-SPN23F/ GdmD**
Wild-type peptiden				
His_6-LtnA2	0-8x Dha/Dhb	N/A	N/A	N/A
His_6-MutA	N/A	0-4x Dha/Dhb	N/A	N/A
His_6-LanA-Spn23F	N/A	N/A	7, 8x Dha/Dhb	7, 8x Dha/Dhb+ decarboxylation
Fusie peptiden				
His_6-LtnA2-NisA	3x Dha/Dhb	N/A	N/A	N/A
His_6-Mut-LtnA1	N/A	2-4x Dha/Dhb	N/A	N/A
His_6-Mut-NisA	N/A	1, 4x Dha/Dhb	N/A	N/A
His_6-Mut-ltnA2	N/A	2, 7-8x Dha/Dhb	N/A	N/A

1x Dha/Dhb hoeveelheid dehydraties gedetecteerd in het peptide door MALDI-TOF MS
X geen peptide gedetecteerd middels MALDI-TOF MS
N/A niet van toepassing/niet bepaald

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